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Fungal hydrolases in the haemolymph of mycosed insects and their roles in pathogenesis

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**FUNGAL HYDROLASES IN THE HAEMOLYMPH
OF MYCOSED INSECTS AND THEIR ROLES IN PATHOGENESIS**

Submitted by Yuxian Xia for the degree of Ph.D

University of Bath

2000

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Dedicated with love to my dear wife, Deyu.

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ABSTRACT

Organic phosphates (e.g. glucose phosphate) and trehalose are present in high concentration in the haemolymph of hosts of the entomopathogenic fungus *Metarhizium anisopliae*, including the tobacco hornworm, *Manduca sexta* and the desert locust *Schistocerca gregaria*. These metabolites are potentially key sources of nutrition for pathogenic fungi. This thesis establishes the production of acid phosphatases (AcP) [EC 3.1.3.2] and trehalases [EC 3.2.1.3]/ α -glucosidases [EC 3.2.1.20] by *Metarhizium anisopliae* *in vitro* and *in vivo* and explores their role in hydrolyzing organic phosphates and trehalose respectively during mycosis.

Injection of saline and β -1,3-glucan caused significant increases in haemolymph AcP in locusts. AcP activity also increased after inoculation with the entomopathogenic fungus *M. anisopliae* var *acridum*. Isoelectric focusing (IEF) was used to separate AcPs in locust haemolymph from different treatments. The results were consistent with the secretion of fungal enzymes and suppression of key host immune-related AcPs during infection.

A comparable study on *Manduca* also provided evidence for fungal secretion of AcPs during mycosis. Dot blot and western blot analyses using antibodies raised against *in vitro* *Metarhizium* AcP suggested that at least one fungal isoform was present in infected haemolymph. This enzyme had similar MW (ca.44.0 kDa) to the *in vitro* form.

Phosphate concentration in the haemolymph of *M. sexta* is a limiting factor for growth of *Metarhizium*. Therefore the increase of phosphate concentration that coincided with and

appeared follow the appearance of *Metarhizium* ACPs in caterpillars could promote fungal growth. .

Elevated haemolymph glucose and trehalose hydrolyzing activity occurred also in haemolymph of mycosed *M. sexta* larvae. IEF separation and characterization of substrate specificity of these enzymes revealed seven trehalases/ α -glucosidases. At least one of these enzymes (pI 4.8) was similar to an *in vitro* fungal form. These results suggest that a *Metarhizium* α -glucosidase may be produced *in vivo* during pathogenesis of *Manduca*. Hydrolysis of trehalose appeared to be excess to the requirements of the fungus. The resulting pathological increase in haemolymph glucose may be detrimental to the host. Not least it may account for the decrease in feeding that occurred in infected insects.

A neutral trehalase gene (*ntl1*) was isolated from a λ EMBL3 genomic library of *Metarhizium anisopliae* ME1. *ntl1* encodes a protein of 737 amino acids with a calculated molecular weight of 83.1kDa. A cAMP dependent-phosphorylation consensus site and a putative calcium-binding site exist in the amino-terminal domain, which suggests the neutral trehalase is a regulatory enzyme. The enzyme has extensive homology with other neutral trehalases from fungi. The *ntl1* gene was expressed in the culture of cell-free haemolymph of *M. sexta* larvae and in the early stages of infection, suggesting the neutral trehalase may be involved in utilisation of trehalose from haemolymph following direct uptake, or through the mobilisation of fungal trehalose reserves.

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Abbreviations

ca.	about
BSA	bovine serum albumin
ConA	concanavalin A
Da	dalton
EPPS	n-[2-hydroxyethyl]piperazine-N'-[3-propanesulphonic acid]
g	gram
IEF	isoelectric focusing
l	litre
μ	micro
m	milli
M	molar
MES	2-[N-morpholino] ethanesulphonic acid
min.	minutes
Mr	molecular weight
OD	optical density
PAGE	polyacrylamide gel eletrophoresis
PBS	phosphate buffer saline
PBST	PBS with tween 20
PBSTM	PBS with skimmed milk power
PEG	polyethylene glycol
pI	iso-electric point
pNPP	p-nitrophenylphosphate

RH	relative humidity
rpm	revolutions per minute
SAD	Sabouraud dextrose agar
SDS	sodium dodecylsulfate
Tris	Tris(hydroxymethyl) aminomethane
w/v	weight for volume

CHAPTER ONE

GENERAL INTRODUCTION

Pressure to minimise synthetic chemical insecticides in insect control has led to an considerable interest in the use of biological agents for the control of insect pests. As key regulators in insect populations, entomopathogenic fungi have great potential as biological control agents.

1.1 The prospects of biological control

The human population is large and still expanding. The global population will rise to at least 8 billion by 2025, a rate of growth demanding an annual increase of 3% in agricultural production (Hulse, 1993). That means the food production must double in next 30 years. It is the great challenge of the early 21st century to produce sufficient and safe food to feed the huge increasing population with less land, declining natural resources and less dependency on chemical pesticides. Therefore, commercial and subsistence farming systems must be highly productive as well as sustainable and non-polluting.

At present, crop protection is based mainly on chemical pesticides. Problems associated with using pesticides include failure of pest control, contamination of the environment, and damage to human health. Concern over these issues has encouraged many countries to seek to reduce pesticide use and has led to a renewed interest in the use of biological

control. In the USA the actual or impending cancellation of some organophosphate and carbamate insecticides that have either lost patent protection or are not being re-registered in many markets because of the Food Quality Protection Act of 1996, has opened new opportunities for biopesticides (Immaraju, 1998). Biological control may be defined as “the use of natural or modified organisms, genes or gene production to reduce the effects of undesirable organism (pests), and to favour desirable organisms such as crops, trees, animals, and beneficial insects and microorganisms.” (NAS, 1987). The transfer of genes from natural enemies and antagonists to crop plants, has the potential to become the major component of pest management of the 21st century, with no significant compromise in the quantity or quality of plant or animal products, the environment, or the expectations of society (Cook, 1993).

However, biological control agents (BCA) account for < 1% of the total pesticide sales (Lisansky, 1997). The majority of BCAs are products relating to the bacterium *Bacillus thuringiensis* (Feitelson et al., 1992); the percentage of the market share of these and other BCAs is likely to increase in the future (Lisansky, 1997). BCAs represent an enormous and still largely untapped natural biological and genetic resource (Cook, 1991).

1.2 Entomopathogenic fungi as biological control agents

As the first recorded pathogen of insects, fungi have been known for a long time to cause dramatic epizootics in insect populations, demonstrating the potential of fungal pathogens for insect control.

Unlike other entomopathogenic micro-organisms, fungi rarely invade the host via the gut. Entomopathogenic fungi predominately invade their host by penetration of the external skeleton or cuticle. Most fungi are non-entomopathogenic because of their inability to degrade cuticle or overcome cuticle-based host defence (Charnley and St. Leger, 1991).

So far, more than 700 species spanning over 100 genera have been shown to be entomopathogenic, mostly Deuteromycotina and Entomophthorales (Roberts and Humber, 1981; Wraight and Roberts, 1987). Some Deuteromycotina species such as *Metarhizium anisopliae* and *Beauveria bassiana* have received most attention as they are relatively easy to culture and have a broad host range (Taper et al., 1986; Charnley, 1997a).

Mycoinsecticides have been using world-widely to control many insect species. *M. anisopliae* is produced on a comparatively large scale in Brazil for control of a number of pasture and sugar cane insects, such as the spittlebug *Mahanarva postica*. *B. bassiana* is produced in China to control pine caterpillars, *Dendromliminus* sp, leafhoppers, *Nephotettix* sp and the European corn borer, *Ostrinia nubilalis*. In the countries of the former USSR, *B. bassiana* is used in conjunction with reduced doses of chemical insecticides to control the Colorado beetle, *Leptinotarsa decemlineata* and the codling moth, *Laspeyresia pomonella*. Increasing interest in developing mycoinsectisides has also arisen in developed countries in the past decade. Some entomopathogenic fungi were registered in European and USA. *M. anisopliae* was registered by the USA Environmental Protection Agency in 1993 for cockroach control, and in 1995 for termite control, and registration packages for use of *B. bassiana*, another imperfect fungus, were recently

approved for grasshoppers and whiteflies (St Leger and Roberts, 1997). *Verticillium lecanii* was registered in Europe for aphid and whitefly control (Hajek and Roberts, 1992).

The application of mycoinsecticides has some advantages over other microbial insecticides (Tanaka and Omura, 1993; Hajek, 1997; St. Leger and Roberts, 1997; Tengerdy and Szakacs, 1998). Fungi form the most important and the best developed group of microbial pathogens of locusts and grasshoppers (Goettel et. al., 1995). Their preference for the external cuticle instead of the gut as the invasion site makes them suitable candidates for the control of Hemipteran pests whose sucking mouth parts usually preclude pathogen uptake (Charnley, 1989a). In addition, many fungal pathogens infect Coleopteran pests, a group with few known bacterial and viral pathogens (Hajek and ST Leger, 1993). Furthermore, most entomopathogenic fungi, including *M. anisopliae*, are not infective or toxic to vertebrates (Siegel and Shaddock, 1990; Saik *et al.* 1990).

Modern formulation techniques, such as incorporating the fungus in mineral or vegetable oils, allow use in dry environments previously thought impossible for fungi (Bateman et al., 1993). Lastly understanding of the process of pathogenesis is rapidly improving and should lead to the development of more virulent mycoinsecticides.

Slow speed of kill and inconsistent results compared with chemical insecticides reduce the effectiveness of mycoinsecticides. So, for commercial purposes, one of the main traits for strain improvement is virulence. However, without the knowledge of the determinants of pathogenicity and virulence strain selection and improvement are restricted to an

empirical rather than to a rational approach (Charnley, 1984). During the last decade, our understanding of the mechanisms of fungal pathogenicity in insects has improved substantially, and several virulence factors have been identified (St Leger, 1995; Clarkson and Charnley, 1996), providing useful parameters for strain selection and targets for genetic engineering.

Pathogenicity characteristics show considerable intraspecific variation. This strain diversity described at the biochemical level within some cosmopolitan species such as *B. bassiana* and *M. anisopliae* (St. Leger et al., 1992a) as well as within species complexes such as *Entomophthora maimaiga* (Hajek et al., 1990), indicates that there is a great potential for manipulation of pathogenicity.

The development of gene cloning and transformation techniques for filamentous fungi in recent years makes possible molecular approaches to strain improvement and the elucidation of pathogenicity. Several genes that encode cuticle-degrading enzymes from entomopathogenic fungus have been cloned, including proteases of *M. anisopliae* (St. Leger et al., 1992b, Smithson et al., 1995, Joshi et al., 1997) and *B. bassiana* (Joshi et al., 1995). Genetic transformation systems have been established in several laboratories (Bernier et al., 1989; Bogo et al., 1996; Barreto et al., 1997; Furlaneto et al., 1999). Multiple copies of PR1 protease gene have been transformed into the *M. anisopliae* genome behind a constitutive promotor. The killing power of the transformants overexpressing PR1 was substantially increased (St. Leger et al., 1996).

1.3 Interactions between insects and their fungal pathogens

Although lots of substantial questions remain to be answered, a great deal of progress has been made in understanding the interactions between insects and their fungal pathogens during last 20 years. There are several reviews in this research area (see e.g Charnley, 1984, 1989a; Charnley and St. Leger, 1991; Hajek and St. Leger, 1994; Clarkson and Charnley, 1996; Charnley, 1997a).

Fungal pathogens: infection process

The invasion strategy of entomopathogenic fungi may be summarised as follows: adhesion of conidia to the cuticle surface, germination, growth of germ tubes and differentiation into appressoria, formation of penetration pegs and penetration of the epicuticle, growth through the procuticle, penetration of the epidermis and invasion of the haemolymph, prior to death of the host (Zacharuk, 1970). Fungal penetration of intact insect cuticle appears to be by a combination of mechanical force and enzymatic degradation, the relative contribution of the two components depending on the structure and composition of the cuticle encountered (Charnley, 1984).

Pathogenesis is initiated by adhesion of a fungal spore to the insect cuticle. The adsorption may involve electrostatic forces (Fargues, 1984), enzymes, lectins and hydrophobic bonding (Boucias et al., 1988). Hydrophobins, hydrophobic proteins found in the fungal cell wall of *M. anisopliae* (St. Leger et al., 1992e) and other fungal pathogens (Talbot et al., 1993) may play a key role in forming these hydrophobic interactions. They are also known to confer water repellency to the spore surface which is necessary for

effective spore dispersal (Wessels, 1993). Pregermination swelling occurs followed by secretion of adhesive mucus that facilitates adhesion (Boucias & Pendland, 1991).

Following successful adhesion, spore germination is triggered by a combination of high humidity and the presence of optimal levels of nutrients (Charnley & St. Leger, 1991; St. Leger et al., 1994a), and the absence of inhibitory compounds (Smith and Grula, 1982). The virulence of fungal entomopathogens is frequently correlated with rapid germination and growth rate (St. Leger, 1991b).

The penetration of insect cuticle often requires, in addition to germination, the precise differentiation of an organised series of structures, which function to concentrate physical and chemical energy over a small area so that ingress may be achieved effectively. For example, germ-tubes of *M. anisopliae* develop an appressorium, the organ of penetration. The appressoria forms a narrow infection peg which breaches the epicuticle to produce (in the procuticle) penetrant hyphae and lateral expansions (penetrant plates) which fracture the cuticle and secrete cuticle - degrading enzymes, facilitating penetration of the cuticle (St. Leger et al., 1992c). Penetration, if not by infection pegs produced from the outside of appressoria, has been known to occur by direct entry of germ tubes (Zacharuk, 1981).

Insects with heavily sclerotised body segments are usually invaded via arthrodial membranes or spiracles (Charnley, 1989a). Germ tubes differentiate into appressoria only after receiving the correct chemical and topographical signals. When the surface topography of the cuticle is incorrect, the fungus seems to grow extensively over the

cuticle surface before producing appressoria, clearly searching for correct location for penetration (St. Leger et. al., 1991c).

Fungi produce a wide range of cuticle-degrading enzymes with activity towards the main chemical constituents of insect cuticle (namely, lipids, proteins and chitin) to facilitate host penetration. The individual groups of enzymes are synthesised sequentially on the basis of the location and availability of the cuticle components that are their substrates i.e. the esterases and proteolytic enzymes (endoprotease, aminopeptidase and carboxypeptidase) are produced initially, followed by N-acetylglucosaminidase, chitinase and lipase (St. Leger et. al., 1986b).

Proteolytic enzymes: Protease was detected histochemically on conidia and during all stages of germination. High level of protease was associated with the formation of large numbers of appressoria and was detected on the *M. anisopliae* infected wings of *C. vomitoria* (St. Leger et.al. 1987a), indicating protease involvement in penetration. In addition to degradation of cuticle proteins, the proteases enable other cuticle-degrading enzymes, particularly chitinases, to gain access to their substrates (St Leger et. al., 1986b). Entomopathogenic fungi produce a number of cuticle-degrading proteases on comminuted cuticle *in vitro*. Extracellular endoprotease activity in culture filtrates of *M. anisopliae* grown on cockroach cuticle have been resolved into three main components, subtilisin-like proteinases (PR1), a thermolysin-like metalloproteinase and trypsin-like serine protease (PR2) (St. Leger et. al., 1994b). Ultrastructural studies with gold - labelled antibodies prepared against PR1 and PR2 demonstrated the presence of these enzymes in the cuticle of mycosed insects (Goettel *et al*, 1989a; St Leger *et al*, 1996a). Furthermore these

studies revealed that while penetration of the epicuticle is primarily by enzymic degradation, penetration of the procuticle involves both enzymic degradation and the mechanical separation of the lamellae (Goettel et. al., 1989b).

PR1 may have a role in isolate pathogenicity (St. Leger, 1995) and specificity (Gillespie et al, 1998). Four subtilisins are produced by *M. anisopliae* which are the product of at least 2 genes. PR1a and PR1b show similar physical and chemical properties (St Leger *et al*, 1994b), the primary amino acid sequences of their respective amino acid termini show only 54% homology (Joshi *et al*, 1997). Both enzymes appear equally effective at degrading a heterogeneous mix of insect cuticle proteins suggesting that they have the potential for non-specific protein degradation during pathogenesis. These enzymes differ in terms of positional specificity for particular amino acids in a protein. In addition to being ultrastructurally located during penetration *in vivo* by *M. anisopliae* through the cuticle of *Manduca sexta* (Goettel et. al., 1989b), PR1a is produced in large quantities by appressoria *in vitro* (St. Leger et al., 1989) and *in vivo* (Goettel et. al., 1989b). Mortality was retarded in larvae of *M. sexta* when conidia of *M. anisopliae* were applied simultaneously with either a specific inhibitor of PR1 (Turkey egg white inhibitor) or a PR1 polyclonal antibody (St Leger et al., 1988a). PR1-like activity is produced by all isolates of *Metarhizium spp.* so far observed and a PR1a null mutant of *M. anisopliae* showed significantly reduced virulence against larvae of *M. sexta* (St. Leger, 1995). Genetically engineered *M. anisopliae* overexpressing PR1 resulted in quicker kill speed of *M. sexta* (St. Leger et. al., 1996c), further confirming that PR1 is a pathogenicity determinant.

M. anisopliae trypsin-like PR2 occurs as multiple isozymes with high activity against solubilised (extracted) cuticle proteins but with little activity against covalently bound (insoluble) cuticle proteins (St. Leger et. al, 1987b; St. Leger et. al, 1994b), probably owing to their poor adsorption onto this substrate (Bidochka & Khachatourians, 1992). Like PR1, the catalytic efficiency of PR2 can be influenced by subsite residues at a distance from the cleaved site (St. Leger et. al., 1987b). Although PR2 has considerably less cuticle-degrading ability than PR1, the products of limited locust cuticle digestion induce PR1 *in vitro* (Paterson et al., 1994). PR2 usually appears in culture 24h before PR1 (Gillespie et al., 1998, Kershaw, 1993). A *M. anisopliae* enzyme with a trypsin sensitivity, PR4, was classified as a cysteine proteinase by Cole et al. (1993), because of its susceptibility to sulphhydryl reagents.

The metalloproteinases of *M. anisopliae* are active against a wide range of proteins including elastin, gelatin, and insect cuticle. Isoelectric focusing has revealed the presence of up to 3 isoenzymes of metalloproteases in some strains of *M. anisopliae* (St. Leger et al, 1994).

Exo-proteases which presumably have a role in providing nutrition have also been identified. A broad spectrum aminopeptidase of *M. anisopliae* has optimal activity for alanine, the most common amino acid in cuticles, and a post-proline dipeptidyl peptidase IV (a serine hydrolase) removes X-prolyl group. Casein-grown cultures contained additional isozymes with activity against lysyl-alanine-X groups (St. Leger et. al., 1993). Aminopeptidase isozymes are sensitive to typical inhibitors of metalloproteinases (St. Leger et. al., 1993; St. Leger et. al., 1995). In addition, *M. anisopliae* produces

carboxypeptidase (St. Leger et al., 1994b; Joshi et al, 1999). Complete hydrolysis of cuticle proteins to amino acids may require the synergistic action of several proteolytic activities. The proteolytic and peptidase enzyme systems of *M. anisopliae* complement each other in splitting most types of peptide bonds (St Leger, 1995).

Several entomopathogenic species secrete multiple proteinases similar to those from *M. anisopliae*, indicating the importance of these enzymes for the growth and survival of these organisms (St Leger et al, 1987b; Joshi et al, 1995).

Chitinolytic enzymes: Chitinolytic enzymes, together with proteases, are the enzymes most frequently considered critical in facilitating invasive mycosis of insects (Charnley and St Leger, 1991). The production of chitinase *in vitro* (St. Leger et al., 1986d) and *in vivo* (St. Leger et al., 1987a) was slow, this is probably due to the fact that chitinase is an inducible enzyme (St. Leger et al., 1986d). Ultrastructural immunocytochemistry studies show that endochitinase of *M. anisopliae* is secreted into cuticle during penetration of the host (*M. sexta*) cuticle, and the release of chitinase is dependent on the accessibility of its substrate (St. Leger et al, 1996a). It seems likely that chitinase plays only a small part in cuticle penetration. They probably have a bigger impact *post mortem* when the fungus grows back out of the cadaver and sporulates on the surface.

Purified chitinase from culture filtrates of *M. anisopliae* grown on 1% ground chitin failed to hydrolyze aryl-glycosides or cellobiose, showed only trace activity against chitotriose, but rapidly degraded chitotetraose. Hydrolysis of crystalline chitin produced N-acetylglucosamine (NAG) (St Leger et al, 1991a).

N-acetylglucosaminidase of *M. anisopliae* had substantial activities against phenol acetylglucosamine, as well as chitobiose, chitotriose and chitotetraose, the major product in each case being NAG (St Leger et al, 1991a).

Isolates of *M. anisopliae* and *B. bassiana* produce multiple extracellular chitinase isozymes (St. Leger et al, 1993b). The endochitinases are likely to be the most effective at solubilizing cuticle polymers and assisting penetration. The exochitinases may function to further degrade oligomers released by endochitinases, resulting in chitobiose and NAG, which can be taken up for nutrition (St. Leger et al, 1996a).

Lipolytic and esterolytic enzymes: The disappearance of the wax layer beneath appressoria of *M. anisopliae* on click beetle cuticle suggests the action of esterase and /or lipase (Zacharuk, 1970), and esterase was extracted from blow fly wings infected with *M. anisopliae* (St. Leger et al., 1987).

Defense system of host insect

Insects are continuously exposed to potentially pathogenic microorganisms and eukaryotic parasites, but only a few encounters result in infection. Insects possess a complex and efficient system of biological defense against pathogens and parasites. This system involves the following: (a) the integument and gut as physical barriers to infection, (b) coordinated responses of several subpopulations of haemocytes when these barriers are

breached, and (c) the induced synthesis of antimicrobial peptides and proteins, primarily by the fat body (Gillespie and Kanost, 1996).

The role of the exoskeleton in host defense

The insect exoskeleton provides a substantial barrier to potential pathogens. Bacteria and viruses are unable to penetrate intact insect cuticle, but some fungi have evolved the ability to infect insects *via* cuticle.

The epicuticle (1-2 μ m), the outer layer of the cuticle, comprises two to four layers, including the inner layer, cuticulin layer, wax layer and a cement layer (Filshie, 1982). It consists mainly of tanned proteins, lipoproteins, lipids, phenolic compounds and hydrocarbons, but no chitin. It provides both chemical resistance and hydrophobic properties (Locke, 1984).

Beneath the epicuticle, the insect procuticle ($\leq 200\mu$ m) comprises chitin fibrils embedded in a protein matrix, together with lipids and quinones (Neville, 1984). Proteins are the major components of the cuticle, usually more than 50% of the dry weight of cuticle, some cuticle can contain 70% protein. Chitin constitutes 17-50% of the dry weight of insect cuticle; more pliant cuticles have a higher chitin content than stiff cuticles (Hillerton, 1984). The main chitin fibrils are laid down parallel to the cuticular surface and as such present a potential barrier to penetration by entomopathogenic fungi (St. Leger et al, 1991). The hardness of the cuticle is dependent on the extent of tanning or sclerotisation (cross-linking by aromatic compounds such as N-acetyldopamine) of the

proteins. Other mechanical properties depend on the relative proportions of protein and chitin and the degree of hydration of the proteins.

The insect epidermis is a sheet of cells polarised for export of materials to the outside toward the cuticle. At the basal surfaces (towards the haemocoel), the plasma membrane is often enfolded to encrease the surface area for adsorption of materials from the haemocoel. The lipids and waxes in the epicuticle are secreted by the epidermal cells through the pore canals.

The epicuticle and procuticle can act as a barrier to infection in several ways. Prophenoloxidase is produced in the haemolymph and transported from plasma to cuticle via epidermal cells and is responsible for the production of quinones at a site of injury (Ashida and Brey, 1995). Phenols, quinones, melanin (the polymerisation product of quinones) and lipids in the cuticle may be inhibitory to fungal germination and growth (Smith & Grula, 1981). Fungal penetration of insect cuticle often results in the deposition of oxidised phenols around the hyphae by the host. Fungal growth and the activity of cuticle-degrading enzymes of *M. anisopliae* were reduced significantly on melanised cuticle (St. Leger et. al., 1988b).

The gut barrier to fungal infection

The gut is seldom used as a primary route of fungal infection. The gut is divided into three sections including the foregut, midgut and hindgut. The foregut and hindgut are of ectodermal and are lined with cuticle, the composition of the cuticle is thought to be

similar to that of the external cuticle apart from the absence of the outer wax layer consistent with the requirement for permeability (Bignell, 1984). Most of the gut cuticle remains unhardened due to the absence of phenol cross-links between the protein molecules (Bignell, 1984). The cuticle is absent in the midgut (Bertram and Bird, 1961), instead midgut epithelium is protected by the peritrophic membrane (pm) which comprises a chitin fibril network set in a protein-carbohydrate complex (Richards and Richards, 1977). In addition to protecting the gut wall from abrasion, the pm provides some protection against pathogenic microorganisms (Brandt et. al., 1978)

Peristalsis and a rapid food throughput prevent the establishment of insect pathogenic fungi which may otherwise be unaffected by their host's gut (Dillon and Charnley, 1991). However, secondary chemicals in the diet of phytophagous insects, antifungal agents produced by metabolism in the gut and fungal antibiotics synthesised by the gut bacterial flora offer significant protection to some insects from fungal invasion (Dillon and Charnley, 1991).

Blood-borne defenses to fungal invasion

Haemolymph (blood) is a complex aqueous medium containing many small molecules and proteins in addition to several classes of haemocytes (blood cells). The major cellular responses of insects to microbial invasion are phagocytosis and encapsulation. The primary response of haemocytes to small particulates, such as bacteria is phagocytosis, a process that can be envisioned as a specialized form of receptor-mediated endocytosis (Gillespie et al., 1997). Phagocytosis of a particle is a multiple step process: attachment,

recognition, signal transduction, activation of pseudopodium formation, ingestion, and assembly of phagosomes. Both granular cells (GRs) and plasmatocytes (PLs) have been reported to be the predominant phagocytic cells in insects (Ratcliffe and Rowley, 1979; Wago, 1991). Phagocytosis of particles often deforms haemocyte appearance (Gillespie et al., 1997), and the phagocytosis rates of PLs and GRs are different *in vivo* and *in vitro* (Ehlers et al., 1992). Factors of microbial origin such as glucans can increase the phagocytic rate of haemocytes (Huxham and Lackie, 1988). However, the microbial killing mechanisms and digestive pathways of insect haemocytes are not clearly understood. Multicellular haemocytic aggregates (nodules) may entrap a large number of invading organism in an extracellular material (Gillespie et al., 1997). Such nodular aggregates may adhere to tissues and larger nodules may eventually be encapsulated. The formation of haemocyte aggregates that resemble nodules can be induced by lipopolysaccharide, Zymosan, laminarin, or some glycoproteins (Lackie, 1988). When a foreign invading organism is too large to be phagocytosed, it becomes encapsulated by multiple layers of haemocytes and /or a melanin coat. Two types of encapsulation are distinguished in insects: cellular encapsulation, mainly described in Lepidoptera, and melanotic (humoral) encapsulation more typical for Diptera (Götz, 1986). Melanotic encapsulation occurs with or without participation of haemocytes (Christensen and Severson, 1993). In contrast to melanotic encapsulation, which is always associated with phenoloxidase (PO) activity, cellular encapsulation can occur without any sign of melanization. In the development of cellular capsules, GRs are generally assumed to contact a foreign particle and then release chemotactic components that attract PLs, which then form a multicellular sheet several layers thick. The inner layers of the capsule may

then melanize, which may contribute to killing of the entrapped organisms (Gillespie et al., 1997).

However, a number of virulent fungal pathogens evade the haemocytes by disguising their presence (modifying the cell wall or removing it all together) or by the production of cytotoxic compounds (see below) (Gillespie *et al*, 2000).

Melanisation is an important component of the cellular defense. As in the cuticle, activation of the prophenoloxidase cascade results in the production of oxidised phenols and melanin, which are toxic to fungi. Protease inhibitors in haemolymph, which have a broad specificity towards fungal proteases (St. Leger, 1991b), may confine prophenoloxidase activation to the site of infection and may also inhibit pathogen proteases (Charnley, 1989a).

A family of antifungal proteins from insect haemolymph has recently been discovered (Landon *et al.*, 2000). Iijima et al. (1993) reported the presence of a constitutive antifungal protein (AFP) in the haemolymph of *Sarcophaga peregrina*. Antifungal proteins called tenecin-3 from haemolymph of *Tenebrio molitor* (Jung et al., 1995) and holtricin-3 from *Holotrichia diomphalia* (Lee et al., 1995) have sequence similarity to AFP. Antifungal peptide, drosomycin, from *Drosophila melanogaster* is not homologous to other insect antifungal proteins, but it does have significant sequence similarity to a family of 5 kDa antifungal peptides isolated from the seeds of Brassicaceae. (Fehlbaum et al., 1995). Lysozyme and cecropin from insects also have antifungal activity (Gillespie et al., 1996).

Fungal growth inside the host:

A successful infection is achieved often when the fungus reaches the epidermis and finally enters the haemocoel (Charnley, 1984), indicating the importance of the cuticular barrier to penetration. Once the fungus penetrates to the haemocoel, it normally grows in a yeast-like phase, viz. blastospores in the haemolymph, which disperse through the insect and invade host tissues; though often tissue invasion occurs at or close to death. Usually tissue invasion begins with the fat body followed by the digestive system, malpighian tubules, hypodermal layers surrounding points of penetration, nervous system, muscles and tracheae (Prasertphon & Tanada, 1968). The host may be killed by profuse growth of fungus, or the production of toxins in the haemocoel which counteract host resistance mechanisms and induce cytotoxic effects.

The production of blastospores with modified wall structure/chemistry within the haemocoel may contribute to the avoidance by the pathogen of cellular host responses (Charnley, 1989a). A failure to do so may result in a more vigorous host response and subsequent failure of the pathogen to kill the host.

Metarhizium produces a series of cyclic depsipeptide toxins called destruxins (DTX). Kershaw *et al* (1999) found a consistent relationship between the titre of DTX production *in vitro* of isolates of *M. anisopliae* var *anisopliae* and their killing power against three insect species; circumstantial evidence that DTX can be a determinant of virulence for *M. anisopliae*. Among the 23 related compounds in the DTX series, DTX A and B tend to predominate and DTX E is generally the most insecticidal. The destruxins affect various

organellar targets, e.g. mitochondria, endoplasmic reticula and nuclear membranes. Interestingly, DTX B has been shown recently to be a specific, dose-dependent and reversible inhibitor of vacuolar-type ATPase, which maintains acidic homeostasis in membrane-bound organelles in eukaryotic cells (Muroi *et al*, 1994). Acidification of intracellular compartments, a pivotal event in many aspects of cell physiology, was also found to be blocked by destruxin B. Inhibition of vacuolar-type ATPases by DTX could account for most, if not all, of the effects of the toxin.

Low doses of DTX A cause tetanic and flaccid paralysis and death in Lepidoptera and adult Diptera; symptoms that are also observed in mycosed caterpillars of the tobacco hornworm. DTXs can also cause dysfunction of the midgut, Malpighian tubules and haemocytes, (Samuels *et. al.*, 1988b). Several studies have shown immunomodulatory effects of DTXs (Vey *et al*, 1985; Huxham *et al.*, 1989; Vilcinskas *et al*, 1997) and inhibition of the cellular immune response in the haemolymph may be the key role of DTX in fungal pathogenesis of insects.

Apart from DTX, *M. anisopliae* also produced the actin antagonists Cytochalasins C and D that induce paralysis in insects upon injection (Roberts *et. al.*, 1992).

Immunosuppression may be a more widespread feature of mycoses in insects. Mazet *et al* (1994) extracted a 10kDa protein toxin from larvae of *Spodoptera exigua* infected with *B. bassiana*. This fungal metabolite inhibited haemocyte spreading, depleted haemocytes and disrupted metamorphosis when injected into naïve larvae.

Finally host death from a fungal infection probably results from a combination of several factors: mechanical damage due to tissue invasion, depletion of nutrient resources and toxicosis. Secretion of antibiotics may help the fungus suppress the saprophytic microflora and take over the cadaver. In conditions of high humidity sporulation occurs on the surface of the cadaver

1.4 Aims of this research

During recent years, special attention has been paid to the extracellular enzymes involved in the penetration of insect cuticle by fungal pathogens. Because the cuticle is the first physical barrier to infection, those extracellular enzymes, including proteases and chitinases may have a particularly important role in host penetration (Charnley and St. Leger, 1991; Clarkson and Charnley, 1996).

However, very little is known about the interactions between insect and pathogen once the fungus has got into the haemolymph. Following one of the few studies on this stage of mycosis, Mazet and Boucias (1996) concluded that *B. bassiana* does not inhibit host protein synthesis and secretes very few exocellular metabolites during the hyphal body stage. However, when the hyphal bodies grow into mycelia that invade host tissue, an event that brings about the rapid death of the host, host protein biosynthesis is suppressed and various metabolites are produced. However, this work may be criticised on two counts. First, one dimensional PAGE separation of haemolymph proteins will not reveal subtle changes in the composition of the haemolymph of mycosed insects. Secondly, the

authors did not extend their observations to other constituents of the haemolymph, including enzymes.

During the process of invasion, fungi obtain nutrients from the insect. Once in the haemocoel the fungus is dependent on the constituents of the haemolymph for growth as tissue invasion takes place either just prior to death or *post mortem*.

A carbon/energy source and phosphorus are just two of the nutrition requirements of both insects and fungi. Carbohydrates in the haemolymph of lepidopteran hosts of *M. anisopliae*, including *Manduca sexta* are in high concentration (Racioppi and Dahlman, 1980) and could satisfy the carbon/energy requirement. Among the carbohydrates, the disaccharide sugar, trehalose, is the predominant haemolymph sugar in many adult insects and the major sugar utilised during flight (Becker et. al., 1996). Thus disaccharides, mainly trehalose, are obvious targets for fungi. Trehalose could be transported intact across the fungal plasma membrane or first be hydrolysed externally and the glucose monomers transported inside and absorbed. The former strategy would need permease and intracellular trehalases, the latter would necessitate extracellular trehalose hydrolases: α -glucosidases and/or acid trehalases.

Many organic molecules in insects, such as glucose, trehalose, amino acids and some proteins, can be phosphorylated. Indeed, organic phosphates comprise a major proportion of the total phosphorus at least in some lepidopteran spp. including *M. sexta* (Pannabecker et al, 1992; Wyatt, 1961). Efficient acquisition and utilisation of phosphorus and the phosphorylated organic molecules requires a ubiquitous class of enzymes known as

phosphatases, which hydrolyse phosphate (Pi) from orthophosphate monoesters (Vincent et al. 1993).

Thus α -glucosidases and acid phosphatases may play an important role in the interactions between insects and fungal pathogens during infection. The potential contribution of these fungal enzymes to pathogenesis of *M. anisopliae* for the tobacco hornworm formed the subject of this research.

CHAPTER TWO

MATERIALS AND METHODS

All chemicals and reagents were supplied by Sigma and were of analytical grade unless otherwise stated. Media chemicals were supplied by Lab M.

2.1 Fungal and insect culture and maintenance

2.1.1 Preparation of conidial stock suspensions

Metarhizium anisopliae var *acridum* (= *Metarhizium flavoviride*, Driver *et al*, 2000) (isolate 330189) (ex *Ornithacris cavroisi*, Orthoptera: Acrididae) and *Metarhizium anisopliae* var *anisopliae* (strain ME1), were routinely taken from liquid nitrogen and streaked onto ¼ strength Sabouraud's dextrose agar medium (SDA; 1% dextrose, 0.25 % mycological peptone, 2% agar, 0.5% yeast extract) and grown for up to 10-14 days at 27 °C in the dark until conidiation. The hydrophobic conidia were harvested in sterile 0.04 % Tween 80 using a glass spreader. Conidial aggregates of spore suspension were dislodged by ultrasonic water bath for 2 minutes. Sterile glycerol was then added to a final concentration of 20% (v/v) and vortexed briefly to get a homogenous stock suspension, then divided into 0.5ml aliquots and flash frozen in liquid nitrogen for storage at - 70 °C.

2.1.2 Preparation of conidia

Conidia were routinely prepared from - 70 °C stock suspensions prepared as above, and streaked onto ¼ strength SDA. Mycelia generally conidiated 6-10 days after inoculation at 27°C. Conidia of *M. anisopliae* var *acridum* were harvested in cotton seed oil or Ondena oil and the resulting spore suspension was filtered through sterile muslin to remove any mycelia. After centrifugation (3,000g for 3 min) spores were resuspended in oil then treated in a sonicating water bath (15°C for 5 min) to break up any aggregates. The concentration of spores was determined using a Neubauer haemocytometer.

Conidia of *M. anisopliae* var *anisopliae* were harvested in 5ml sterile 0.04 % Tween 80. Aggregates were dislodged in an ultrasonic water bath and the number of conidia was determined microscopically.

2.1.3 Maintenance of *Manduca sexta*

The tobacco hornworm *M. sexta* (Lepidoptera, sphingidae) was maintained in an insectary at 25 °C using a 17 hour light and 7 hour dark photoperiod. The larval stages of *M. sexta* were reared on a modified version of Bell and Joachim's artificial diet (Bell and Joachim, 1976). 504g batches of diet pre-mix containing 750g wheat germ, 350 casein, 300g sucrose, 150g dry yeast, 100g wesson's salt mixture, 15g sorbitol acid, 10g cholesterol, 10g Methy-*p*- hydrobenzoate, 10g Choline chloride were blended at a low speed in a kenwood mixer. 1250 ml of boiling distilled water was heated was added to the pre-mix which was then blended for an additional 5 minutes. 45g agar was heated separately in 1250 ml distilled water and subsequently transferred to the blender. This was mixed briefly at low speed and cooled to 70 °C. 12g ascorbic acid, 0.3g vanderzant's vitamins, 12ml 10% formaldehyde, 6ml linseed oil and 6ml vegetable oil was added to the mixture which was blended at low speed until thoroughly mixed. The diet was poured into

containers lined with aluminium foil and allowed to set at room temperature in a flow cabinet. Once set the diet was stored at 4 °C until required.

Newly hatched larvae were placed in individual plastic pots containing 5g artificial diet. These were allowed to grow for 14 -16 days until the 5th and final instar was reached and maintained under the same photoperiod until the larvae reached the “wandering” stage. Wanderers were placed into corked wooden blocks, which were laid on their sides for 10 days until the larvae pupated, these pupae were placed on paper towels until the adult moths emerged. Adults were housed in a separate unit and persuaded to lay eggs on nappy -liners impregnated with macerated tobacco leaves. New eggs were harvested daily in order to maintain the insect line through the cycle described.

2.1.4 Infection of *Manduca sexta*

Early 5th instar *M. sexta* larvae were inoculated with ME1 by dipping the larvae briefly into the conidial suspensions of known concentration or injecting the larvae with 20µl of the conidial suspensions of known concentration. Larvae were starved overnight to allow germination of the conidia (the diet contains antifungal compounds) and then returned to their normal diet and maintained at 25 °C using a 17 hour light and 7 hour dark photoperiod.

2.1.5 Harvesting haemolymph from *M. sexta* larvae

Haemolymph was removed from anaesthetised larvae (chilled on ice for 15 minutes), by making an incision below the “tail”. Larvae were squeezed gently using a pair of forceps

over sterile Eppendorf tubes containing 50µl haemolymph extraction buffer: 50mM EPPS (pH 7.0), 1mM PMSF, 10mM phenylthiourea. Haemolymph samples were stored on ice for periods of up to 24 hours, or stored at - 20 °C for up to 1 month.

2.1.6 Maintenance of *Schistocerca gregaria*

Mature adult males of the desert locust, *S. gregaria* (Orthoptera: Acrididae), were used in all experiments. Locusts were reared according to the method described by Gillespie *et al* (1999) and fed on fresh wheat shoots, wheat bran (supplemented with dried brewer's yeast) and water. The water was periodically treated with a 5% antiprotozoal solution (w/v; 4.26% sodium sulfamethazine; 3.65% sodium sulfathiazole; 3.13% sodium sulfamerazine) to suppress growth of the sporozoan parasite *Malamoeba locustae*. Experimental insects were not treated to prevent pharmacological effects on the immune system.

2.1.7 Treatment of *S. gregaria*

Inoculation with fungus

Locusts were chilled at 4°C for 1 h prior to topical inoculation with an LD₅₀ dose viz. 5 µl of cotton seed oil containing 7.5×10^4 conidia (Bateman *et al.*, 1993) applied under the pronotum using a microsyringe. Control locusts were treated with cotton seed oil alone. Insects were housed individually in an incubator at 28±2°C with a 16h light: 8h dark photoperiod and were fed wheat seedlings.

Injection of laminarin

A hand microapplicator fitted with an all-glass syringe (Burkard Co) was used to inject locusts with the algal β -1,3-glucan, laminarin. Twenty five μ l of 0.5% (w/v) laminarin in Hoyles saline (0.234M NaCl; 0.006M KH_2PO_4 ; 0.004M KHCO_3 ; 0.002M CaCl_2 ; 0.002M MgCl_2 ; 450 mOs/Kg; pH 4.4) were injected through a 15 gauge needle into each locust dorsoventrally in the intersegmental membrane between the 3rd and 4th abdominal segments. Controls were either not injected or injected with saline only. Insects were housed individually in an incubator at $27\pm 2^\circ\text{C}$ with a 12h light: 12h dark photoperiod and were fed wheat seedlings.

Collection and treatment of haemolymph

Haemolymph was collected from the arthrodial membrane of the hindleg of the locust. The membrane was first swabbed with 70 % ethanol, allowed to air dry and then pierced with a sterile needle. The haemolymph was collected using a 10 μ l Eppendorff Pipetman over ice to prevent coagulation.

Haemolymph was diluted 10x with sterile ice cold anticoagulant buffer (AC buffer; 0.098 M NaOH; 0.180 M NaCl; 0.017 M EDTA (free acid); 0.041 M citric acid; 440-450 mOs/Kg; pH4.8). For whole blood assays the diluted haemolymph was ultrasonicated for 20 sec then frozen at -20°C to rupture the haemocytes. Samples were then centrifuged at 13,000 g to remove cellular debris. The supernatant was decanted and stored until use at -20°C . When haemocytes and plasma were required separately, haemolymph in anticoagulant buffer was centrifuged at 3,000 g for 10 min. The plasma supernatant was

pipetted off and stored until use at -20°C. The haemocyte pellet was resuspended in sterile distilled water then ultrasonicated, frozen and centrifuged to disrupt the cell membrane and remove debris as with whole blood samples. The supernatant was pipetted off and stored until use at -20°C

2.2 Measurement of fungal growth

2.2.1 Dry weight of mycelia

100ml basal salt media of varying phosphate concentrations in 250ml flasks were inoculated with 1×10^7 fungal conidia and were shaken at 150rpm at 27 °C. Mycelia were collected four days after the inoculation and dried at 60 °C until constant weight.

2.2.2 MTT method

2.5×10^5 conidia were inoculated into 1 ml cell-free haemolymph of 5th instar *M. sexta* larvae containing 100µg ampicillin and different concentrations of phosphate (adjusted with 1M phosphate buffer, pH 6.5). The inoculated media were placed into evaporation-free cell culture plates. The plates were sealed with parafilm and shaken at 150rpm at 27 °C for 2 days. Fungal growth was determined using the MTT[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as described by Freimoser et al (1999), with respect to a standard curve prepared from 4 day blastospores *in vitro*. The blastospores

were produced by the method of Humphreys et al. (1989). The MTT was added to the medium 16 h before the measurement.

2.3 Haemocyte monolayer preparation and staining haemocytes for acid phosphatase activity

2.3.1 Haemocyte monolayer preparation

Locusts were chilled on ice for 10 min to make handling easier. Haemolymph was collected from the arthrodial membrane of the hindleg of locusts. The membrane was first swabbed with 70% (v/v) ethanol, allowed to air dry and then pierced with a sterile needle. The haemolymph was collected onto a meniscus of Hoyle's Saline on a syringe containing 200 µl of the chilled saline. The syringe was inverted several times to disperse the cells before dispensing 50 µl onto endotoxin-free cover slips. Two cover slips were placed onto an endotoxin-free microscope slide and incubated for 15 min at room temperature in a moist chamber in order to allow the haemocytes to fall out of suspension and adhere to the glass. The monolayers were washed four times with Hoyle's saline to remove plasma and any non-adherent material. Some monolayers were overlaid with 100 µl of 1mg/ml solutions of laminarin and zymosan in Hoyle's saline. All monolayers were subsequently stained histochemically for acid phosphatase activity.

Three types of haemocyte were identified following the nomenclature of Gillespie *et al* (1999). These were: vacuolated plasmatocytes (PLs), which spread on E-toxa-coated glass and contained large numbers of granules and vacuoles; coagulocytes (COs) which

lysed or degranulated *in vitro* and sometimes appeared as a nucleus with remnants of surrounding cytoplasm attached to the slide by thin streamers of diffuse coagulum; granular cells (GRs) which were phase bright, granule-containing round cells anchored by thin filopodia.

2.3.2. Staining haemocytes for acid phosphatase activity

Monolayers were stained for acid phosphatase activity using a Sigma diagnostic kit (181-A, lymphocyte acid phosphatase kit). The assay utilised the simultaneous capture principle to generate an insoluble chromogenic Naphthol AS-BI-GBC complex which is dark red in colour. The staining procedure was carried out according to the manual provided. Monolayers of more than 100 cells were stained and cells that were dark red in colour scored as positive for acid phosphatase.

2.4 Enzyme assay

2.4.1 Acid phosphatase

Acid phosphatase activity was routinely assayed using a colorimetric assay based on the release of *p*-nitrophenyl from *p*-nitrophenylphosphate (pNPP) by the enzyme (Wynne 1994). Culture fluids or haemolymph were centrifuged (15min at 10,000g) at 4 °C. 50µl cell-free supernatant, 200µl acetate buffer (50mM, pH5.0), containing 1 mM Mg²⁺ and 10mM pNPP, were used to assay AcP. The concentration of *p*-nitrophenol formed in the reaction was determined from the absorbance at 420nm on Dynatech MR5000 plate reader, using a calibration curve of *p*-nitrophenol standard solution (5µM---2000µM),

One unit of activity corresponds to 1 μ mol nitrophenol released per minute. Specific activities normalised to protein content were calculated.

For analysis of the substrate specificity of purified acid phosphatases, the phosphatase activity against organic phosphate esters, including glucose-6-phosphate, glycerophosphate, fructose-6-phosphate, p-nitrophenylphosphate, was assayed by the release of inorganic phosphate. The method employed was essentially that described by Ames et al (1960): 0.1ml enzyme solution was mixed with 1.1ml 30mM substrate and 0.3ml 0.2M pH5.0 acetate buffer, and incubated at 27 °C for 1hour. The reaction was stopped by addition of 1.5 ml 10% trichloroacetic acid, and 0.3ml aliquots of the reaction mixture were assayed for inorganic phosphate formed in the reaction. Two blanks were also included, one blank without substrate but containing the enzyme and buffer, the other without enzyme. The inorganic phosphate formed in the reaction was assayed according to the method of Chen et al (1956): 0.70 ml of a mixture of 10% ascorbic acid and 0.42% ammonium molybdate. 4H₂O in 0.5 M H₂SO₄ (1 : 6) was added to 0.30 ml of the reaction mixture, or to 0.3 ml of distilled water (the blank), and incubated for 1 hour at 37 °C. The concentration of inorganic phosphate was determined by measuring the absorbance at 820 nm against a series of known phosphate standards (Chen et al, 1956). One unit of activity corresponds to 1 μ M inorganic phosphate released per minute at 27 °C.

2.4.2 Trehalose-hydrolysing enzyme activity and α -glucosidase assay

Trehalose-hydrolysing enzyme activity was assayed by the amount of glucose released from the hydrolysis of trehalose. α -Glucosidase activity was assayed by the amount of

glucose released from the hydrolysis of maltose. 25 µl of enzyme was incubated with 0.1M substrate (trehalose or maltose) in 20mM EPPS (pH7.0) in a final volume of 50 µl for 30 minutes at 27 °C. The reactions were terminated by thermal denaturation at 100 °C for 5 minutes followed by chilling on ice for 5 minutes to prevent the protein re-folding. The concentration of glucose was then determined using a modification of the glucose oxidase assay (Trinder, 1969). Glucose was first oxidised to glucuronic acid and hydrogen peroxide by glucose oxidase. The hydrogen peroxide formed then reacts in the presence of peroxidase with 4-aminoantipyrine and p-hydroxybenzene sulphonate to form a quinoemine dye, with an absorbance maximum at 505nm. The intensity of the colour produced is directly proportional to the glucose concentration in the sample. 150µl Trinder reagent (sigma) containing 0.5mM 4-aminoantipryine, 20mM p-hydroxybenzene sulphonate, 15,000 units/L glucose oxidase and 10,000 units/L hoseradish peroxidase, was added to the assay and the colour was left to develop at 27 °C for 20 minutes. The concentration of glucose was determined by measuring the absorbance at 520nm against a series of known glucose standards. One unit of enzyme activity was defined as the amount of enzyme required to generate 1mM of glucose per minute.

2.5 Gel staining methods

2.5.1 SDS - PAGE and protein staining

SDS - PAGE was routinely carried out using 12% polyacrylamide gels following methods outlined by Laemmlli (1970) using the Mini - Protean™ SDS - PAGE system

(BioRad). Gels were run at constant 120V until the dye front reached the bottom of the stacking gel, and then at a constant 200V until the dye front reached the bottom of the gel.

The gel was then removed from the Mini - Protean™ apparatus and stained by silver staining method using silver stain kit (Pharmacia). The molecular weight of the protein was determined by measuring its mobility relative to a series of molecular weight standards.

2.5.2 Flat IEF and acid phosphatase activity staining

Isoelectric focusing (IEF) was carried out in 2.2 % flat IEF gels (Pharmacia Biotech). The anode was a strip of Whatman No.3 filter paper soaked in 1M phosphoric acid, the cathode was a strip of Whatman No.3 filter paper soaked in 1M sodium hydroxide. The run was performed in a LKB gel apparatus. The power was regulated as constant 10 W for 3.5 hours. The cell-free supernatants were dialysed (48 hr, 4 °C, against four changes of 200 vol distilled water) before isoelectric focusing.

After isoelectric focusing, the gels were stained for acid phosphatase activity with a stain solution (Nagy et al.1981) for about 20 minutes at room temperature. Stain solution: 150mg 1-Naphthyl phosphate + 50mg Fast Blue RR salt dissolved in 50mM pH5.0 acetate buffer. The staining was terminated by transferring the gel into 7% acetic acid. Two lanes from each run were not stained, one from the middle of the gel and another from one edge of the gel. They were cut into 0.5 cm pieces and each piece was soaked in 1 ml distilled water, frozen for 12 hours at -20 °C, then thawed out it and pH value determined with a pH meter (Phk-200-010E, Fisons.) at room temperature. The relation of pH values to gel

length was determined.

2.5.3 Vertical IEF and α -glucosidase activity staining

Isoelectric focusing was carried out in 5% vertical IEF gels using the Mini - Protean™ apparatus (BioRad), broad pH range ampholine (BioRad, pH3-10) or narrow pH range Rotolytes (BioRad) vertical gels were poured using the Mini - Protean™ apparatus (BioRad). The cathode solution was 2.90g lysine (free base) and 3.48g arginine (free base) dissolved in 1L distilled water; the anode solution was 0.84 ml phosphoric acid in 1L distilled water. The sample solution contained 50% glycerol. The run was carried out in stepwise constant voltages of 100 volts for 1 hour, then 250 volts for 2 hours and 500 volts for 30minutes.

After isoelectric focusing, the gels were stained for α -glucosidase activity using an overlay method according to the following procedure (Manchenk, 1994). Component A [0.1M pH5.0 citrate buffer 10ml, 1000U/ml glucose oxidase 50 μ l, 2500U/ml peroxidase 50 μ l, 25mg/ml 3- amino-9-ethyl carbazole (dissolved in acetone) 2ml, 100mg trehalose (or maltose)] and component B [2% agar solution (60°C)] were mixed and poured over the gel surface. The gel with attached overlay was incubated at 37 °C until red brown bands appeared, then were fixed by transferring the gels into 7% acetic acid.

2.6 Enzyme purification techniques

2.6.1 ConA affinity chromatography

For acid phosphatase purification, crude proteins were applied to a coA- linked Sepharose column (1x15cm) which had been pre-equilibrated with 20mM pH7.0 EPPS wash buffer. Unbound protein was eluted from the column with buffer until no absorbance was detected at 280nm. Bound glycoproteins were eluted using wash buffer containing 250mM α -methyl-D (+)-glucoside. Fractions containing enzyme activity were pooled and the salt removed by dialysis against 100 vol distilled water overnight with 3 times changes of water.

2.6.2 Anion exchange chromatography

For α -glucosidase purification, crude proteins were loaded onto a Sepharose-Q anion exchange column (1x15cm) which had previously been equilibrated with 20mM pH5.5 acetate buffer. The unbound protein was eluted with buffer at a flow rate of 0.5ml/minute. When all of the unbound protein had been eluted (no absorbance detected at 280nm), a 500ml 0.0 M - 0.5 M NaCl gradient was used to remove bound proteins. Fractions containing enzyme activity were pooled and the salt removed by dialysis against 100 vol distilled water overnight with 3 times changes of water.

2.6.3 Gel filtration

Partial purified fractions containing enzyme activity were loaded onto a Sephadex G100 (2.4 x 90 cm) filtrate column, which had previously been equilibrated with 50mM pH5.5 acetate buffer. Proteins were eluted with the same buffer at a flow rate of 1.5ml/minute. Fractions containing enzyme activity were pooled, concentrated by polyethylene glycol

(PEG) 30,000 and the salt removed by dialysis against 100 vol distilled water overnight with 3 times changes of water.

2.6.4 Semi - preparative IEF

Fractions with enzyme activity were concentrated by PEG 30,000 and dialysed against 1000 vol distilled water overnight, then the enzyme solutions were loaded onto narrow range IEF gels. After separation, bands with different pI values were excised after activity detection and were electrically eluted separately into dialysis tubes separately. The purity of each isozyme was identified using SDS-PAGE stained with a silver stain kit (Pharmacia).

2.6.5 Semi-preparative gradient native PAGE

Gradient native polyacrylamide gels were poured using the Mini - Protean™ (BioRad). Semi-purified enzyme was dialysed against 1000 vol of distilled water overnight before loading onto the gradient gel. Gels were run at constant 100V until the dye front reached the bottom of the stacking gel, and then at constant 150V. After separation, each band with enzyme activity was cut off post activity detection and was electrically eluted into dialysis tube. The purity of enzyme was identified using SDS-PAGE stained with silver stain kit (Pharmacia).

2.7 Production of fungal acid phosphatases in liquid culture

M. anisopliae var *acridum* or *M. anisopliae* var *anisopliae* were grown under culture conditions optimised for acid phosphatase production (unpubl): 10^8 conidia in 100

ml of medium (basal salts including 10mg Pi/l, 2% glucose and 2g l⁻¹ NaNO₃, 50mM 2-(*N*-morpholino) ethane sulphonic acid buffer, pH 6.0) in 250 ml flasks, shaken at 150 rpm at 27°C for 4 days. Mycelium was removed by filtration over a sintered glass funnel and the filtrate concentrated ca. twenty - fold by PEG 30,000 at 4°C. The concentrated extract was then dialysed overnight against 100 volume of distilled water with 3 changes of water, then dialysed against 10 volume of 20mM n-[2-hydroxyethyl]piperazine-N'-[3-propanesulphonic acid], pH 7.0 for 5 hours.

2.8 Enzyme purification

2.8.1 Purification of acid phosphatase from *M. anisopliae* var *anisopliae*

Acid phosphatases were purified from filtrates of 4-day cultures grown under optimised culture conditions (see Chapter 4): 10mg Pi/L, pH 6.0, inoculum of 10⁸ spores to 100ml medium in 250 ml flask, shake at 150 rpm in 27°C for 4 days. Mycelium was removed by filtration over a sintered glass funnel and the filtrate concentrated ca. twenty - fold by PEG 30,000 at 4°C. The concentrated extract was then dialysed overnight against 100 volumes of distilled water with 3 changes of water, then dialysed against 10 volumes of 20mM EPPS, pH 7.0 for 5 hours. The dialysed concentrated extract was then used to purify acid phosphatases by affinity chromatography (Con A - Sepharose column) and semi-preparative narrow range IEF (pH5.5 - 8.5).

The separation was performed on a Con A - Sepharose column (1 x 15 cm) equilibrated with 20mM EPPS, pH 7.0. Elution of the bound glycoproteins was done with 250mM α -

methyl-D (+)-glucoside in 20mM EPPS, pH 7.0 at 4°C. The majority of the proteins in the concentrated filtrate did not bind to the column. The eluate was concentrated by PEG 30,000, and then dialysed overnight against distilled distilled water. The dialysed concentrated eluate was loaded onto a narrow range IEF (pH5.5-8.5), typically 12mg glycoproteins per gel. After acid phosphatase activity staining, 6 isoforms were cut off and electrically eluted into dialysis tubes separately and the purity of each isoform was tested on SDS - PAGE using silver staining.

2.8.2 Purification of α -glucosidase from *M. anisopliae*

Culture conditions for purification of α -glucosidase were as follows: 250ml conical flasks containing 50ml of basal salts [1g/l KH_2PO_4 , 0.5g/l MgSO_2 , 50mM MES (pH 6.0)] solution (Cooper and Wood, 1975) supplemented with 2g/l NaNO_3 , 10g/l sorbitol and 10ml/l trace elements were inoculated with 1.0×10^7 ME1 conidia incubated at 27 °C and shaken at 150 rpm for 3 days.

Mycelium was removed by filtration over a sintered glass funnel, and cell wall bound proteins of mycelium were extracted twice at 4 °C for 4 hours by 2 v/w 0.2 M pH7.2 potassium phosphate buffer. The extracts and the filtrate were put into dialysis tubes, and then concentrated ca. twenty - fold by PEG 30,000 at 4 °C. The concentrated extract was then dialysed overnight against distilled water, then dialysed against 10 volumes of 20mM sodium acetate, pH 5.5 for 5 hours. The dialysed concentrated extract was then used to purify α -glucosidases by ion exchange chromatography (Q - Sepharose), Sephadex G100

gel filtration, followed by semi-preparative narrow range IEF (pH 4.5 - 5.1) and native gradient PAGE.

Separation was first performed on a Q - Sepharose column (1 x 15 cm) equilibrated with 20mM sodium acetate, pH 5.5. Elution of acid proteins was achieved with a linear gradient (0 - 0.5 M NaCl) in same buffer with detection at 280nm (Appendix 1). Fractions with α -glucosidase activity were pooled. The second α -glucosidases peak of eluate, fractions 35 – 45, was concentrated by PEG 30,000 and then dialysed against distilled water. The dialysed concentrated α -glucosidases sample was further separated on a 2.4 x 90 cm column of Sephadex G100 (Appendix 2). The major α -glucosidase peak of eluate, fractions 51 - 75, was concentrated by PEG 30,000 and then dialysed against distilled water. This α -glucosidase solution was further separated on narrow range IEF (pH 4.5 - 5.1) with detection of α -glucosidase activity, 2 isoforms were cut off and electrically eluted. The two isoforms were put onto 10-20 % native PAGE for further separation, α -glucosidase band was cut off and electrically eluted after detection of α -glucosidase activity. The final preparations of α -glucosidases produced single bands on SDS-PAGE stained with silver.

2.9 Raising antibodies against acid phosphatase

Three purified acid phosphatase isozymes with alkaline pI values were inactivated by heat for 5 minutes at 95 °C. The solution (70 μ g AcP) was then mixed with Freund's Complete Adjuvant (DifCo). The sample was then given to a licensed animal house

(University of Bath) and used to inject a long eared rabbit. For subsequent boosts, Freund's Incomplete Adjuvant was used. 70µg protein was used to boost the rabbits every 14 days. Two rabbits were used. Test samples of blood were collected regularly and allowed to clot at room temperature for up to 4 hours in glass universal tubes. The tubes were then transferred to 4°C overnight, to allow the clot to detach from the wall of the vessel. The supernatant was then centrifuged at 17,210g for 20 minutes. The serum was then stored with 0.05% sodium azide in aliquots at -20°C. The level of antibody in the rabbit sera was tested regularly, and once no significant increase in antibody response was observed, boosting was stopped and the rabbits were exanguinated and blood collected.

In order to estimate the antibody concentration required for a positive signal, the polyclonals were titrated using a dot blot method. To adsorb and remove antibodies raised against *E. coli* in the adjuvant, *E. coli* phage lysate was bound to nitrocellulose membranes, and then incubated with serum at room temperature, the nitrocellulose membranes were changed every 30 mins for 3 times. The purified antiserum was frozen until needed.

2.10 Dot blotting

2 µl of sample was applied to a nitrocellulose membrane. The sample was baked onto the filter in an oven at 60 °C. When dry, the membrane was re-wetted with two changes of Tris-Buffered Saline supplemented with Tween (TBST - 0.01 M Tris-HCl, pH 8.0, 0.05 % Tween-20 in 1 l dH₂O). Non-specific binding sites were saturated by blocking the membrane with 2 % skimmed milk (Marvel) in TBST for at least one hour at room temperature. The nitrocellulose membrane was then washed for 20 minutes in four

changes of TBST. Membranes were covered with antisera diluted as appropriate with TBST (without Tween-20) supplemented with 0.2 % skimmed milk and incubated for 1 hour at room temperature with gentle shaking. Membranes were then washed in four changes of TBST over 20 minutes before transfer into the secondary antibody linked to horseradish peroxidase (1:1000 dilution in TBS plus 0.2% milk). Membranes were incubated at room temperature for 1 hour and then washed, as before in 4 changes of TBST before developing colour with a solution of 9 ml TBS, 1 ml chloro-1-naphthol (from a stock of 3 mg ml⁻¹) and 10 µl H₂O₂ (30 %). If colour was not apparent after 10 minutes, a further 10µl H₂O₂ was added. Membranes were allowed to dry and stored in foil to protect from light. For increased sensitivity the Vectastain® ABC kit (Vector Laboratories) was used for detection according to the manufacturer's instructions. 3,3' - diaminobenzidine tetrahydrochloride (DAB, sigma) was used as the chromogenic substrate for the peroxidase.

2.11 Western blotting

After protein separation, SDS-PAGE gels were equilibrated for 10 minutes in transfer buffer: 25mM Tris, 192mM glycine, pH8.3 with 20% Methanol and 0.05% SDS. Electroblothing was carried out in transfer buffer for 1.5 hour at 300mA using the Mini-Protein™ electroblotting system (BioRad) and nitrocellulose membranes (Hybond™ - C extra, Amersham). Once transfer was complete, membranes were rinsed immediately with distilled water for 10 minutes with gentle shaking, then stained the membranes with Ponceau S to locate the molecular weight markers. The position of bound antibody was located using Vectastain ABC kit (Vector), according to the manufacturer's instructions.

3,3' - diaminobenzidine tetrahydrochloride (DAB, sigma), substrate for peroxidase, was used as chromogen.

2.12 Protein determination

Protein was routinely determined by the Bradford method (Bradford, 1976) using a protein assay kit (Bio-Rad, U.K.). A standard curve was generated each time using a stock solution of bovine gamma globulin. The absorbance of samples was measured at 595nm.

2.13 Statistical analysis

Statistical comparisons of data were performed using Student's *t* test.

2.14 Molecular techniques

2.14.1 Screening genomic library and RT-PCR of full length cDNA

Two degenerate sense primers (ntreF1/ntreF2) and one degenerate antisense primer (ntreR) were designed to correspond, respectively, to two regions conserved in neutral trehalases from yeasts and filamentous fungi (Table 2.1). However, only the combination of sense primer ntreF1 and an antisense primer ntreR generated a product of the predicted size. Using these two primers, a 1.0kb fragment was amplified by PCR from genomic DNA of ME1. The 1.0kb PCR product was cloned and sequence comparison confirmed that it was a fragment of a neutral trehalase gene. The deduced amino-acid sequence revealed significant identity to corresponding regions of neutral trehalases from yeast and filamentous fungi. The 1.0kb PCR fragment was used to screen an λ EMBL3 genomic

library of *M. anisopliae* DNA that was previously prepared in our lab. A 5.8kb XhoI fragment hybridising to the 1.0kb fragment was subcloned into pBluescript. The nt sequence of a neutral trehalase gene in the subclone was determined by overlapping starting from the two ends of the 1.0kb sequence.

Two primers, ntreCF and ntreCR (Table 2.1), based on the sequence around the start code (-6nt — +14nt, sense) and the last 21nt (antisense) of the genomic sequence of *Man11* gene were designed for RT-PCR to amplify the full length cDNA of the neutral trehalase using mRNA preparation. RT-PCR was carried out using the Reverse Transcription System according to the manufacturer's instructions (Promega). The amplification protocol was as followings: First, RT was at 48 °C for 45min, then a denaturation step at 94 °c for 2min followed by 30 cycles of the follows: denaturation at 94 °C for 30s, annealing at 60 °C for 1min, extension at 68 °C for 3min. A last elongation step was carried out at 68 °C for 7min. Amplification products were cloned using the TA cloning kit according to the supplier's instruction (Promega).

2.14.2 DNA manipulations

Routine DNA and RNA manipulations were essentially performed according to Sambrook *et al.* (1989). Plaque lifts, Southern hybridisation were performed using Hybond-N membranes (*Amersham*) according to the manufacturer's instruction. Oligonucleotides used in this study were obtained from *Life Technologies* and are listed in Table1. Plasmid preparations were carried out using Qiagen minipreps. Calcium/Manganese-treated *E. coli* were transformed using the protocol of Hanahan *et al.* (1991).

Transformation of yeast was carried out by the alkali cation procedure with lithium acetate and polyethylene-glycol (Ito et al. 1983). DNA sequencing in both directions was performed using terminator dye technology on an ABI 377 automated Sequencer (PE Applied Biosystems). DNA and deduced protein sequences were analysed by the GCG program. DNA and protein homologies were sought in peptide sequence database using the BLAST network service.

2.14.3 PCR amplification of the *ntl1* gene

The genomic DNA of *M. anisopliae* prepared according to the method of Raeder and Broda (1985) was used as templates to amplify a 1.0kb segment of gene encoding a neutral trehalase. Two sense primers (ntreF1/ntreF2) and a antisense primer (ntreR, Table1) were based on amino sequences conserved in neutral trehalases from yeasts [*Saccharomyces cerevisiae* putative trehalase-encoding gene (Nwaka et al., 1995), *S. cerevisiae* neutral trehalase gene (Kopp et al., 1993); and *Kluyveromyces lactis* neutral trehalase gene (Amaral et al., 1997)], and neutral trehalases from filamentous fungi [*Magnaporthe grisea* neutral trehalase gene (Sweigard et al., 1997), *Emericella nidulans* (*Aspergillus nidulans*, d'Enfert et al., 1999)]. Only the combination of sense primer ntreF1 and a antisense primer ntreR generated the right product. The amplification protocol consisted of a denaturation step at 94 °C for 4 min, followed by 35 cycles of the following steps: denaturation at 94 °C for 30s, annealing at 63 °C for 1min, extension at 72 °C for 3min. A last elongation step was carried out at 72 °C for 7 min. Amplification products (1.0kb) were subcloned using the TA cloning kit according to the supplier's instruction (Promega).

2.14.4 Southern analysis

Twenty micrograms of ME1DNA were digested separately with BamHI, EcoRV, NotI, SmaI and XhoI. The digests were separated on a 1.0 % agarose gel and blotted onto Hybond-N membrane filter (Amersham). The filter was probed with the 1.0kb PCR product amplified from ME1 DNA with primers ntreF1 and ntreR. The probe was labeled by Ready-To-Go DNA labeling kit (Pharmacia Biotech). Hybridization conditions were fairly stringent (65 °C), the final wash conditions were 0.1×SSC, 0.1% SDS at 60 °C.

2.14.5 RNA and mRNA preparation

Mycelia were collected by filtration. Cells from infected and non-infected *M. sexta* haemolymph were collected by centrifugation at 6,000 ×g at 4 °C for 10 mins, frozen in liquid nitrogen, and disrupted by manual grinding in the presence of liquid nitrogen. Total cell RNA was prepared using the RNeasy® total RNA isolation system (Qiagen) following the instructions supplied with the addition of an extra phenol/chloroform extraction. RNA quantification in different samples was achieved both by UV adsorption and by running an aliquot on MOPS/formaldehyde gel (Ausubel *et al.*, 1992). mRNA for RT-PCR was prepared from 100µg total RNA using PolyATtract® mRNA Isolation Systems (Qiagen) according to the instructions.

2.14.6 Expression of the *Ma ntl1* gene in yeast

The *ntl1* gene was expressed under GAL1 promoter in plasmid pYES2 (Invitrogen). Neutral trehalase PCR fragment generated from cDNA using two primers (ntreEF and ntreER) was constructed into the multiple cloning site of pYES2 vector. The neutral trehalase gene was inserted into BamHI and XhoI sites. The junctions were confirmed by sequencing. The construct was transformed into *S. cerevisiae* strain INVScI (Invitrogen). The transformants were selected for URA3 prototrophy and neutral trehalase expression.

2.14.7 Analysis of the expression of the *Ma ntl1* gene in *S. cerevisiae*

The transformants were grown in a shaker at 30 °C in YPD (1% yeast extract, 2% peptone and 1% glucose) or in an inducing YPGal media (1% yeast extract, 2% peptone and 1% galactose) for 3 days. Crude enzyme extracts were prepared from equal wet weight amounts of cells using glass beads. Neutral trehalase activity was assayed by measuring the amount of glucose released from the hydrolysis of trehalose. 25 µl of enzyme extract was incubated with 0.1M trehalose in 20mM MES pH6.0 in a final volume of 50µl for 1hr at 30 °C. The reactions were terminated by thermal denaturation at 100 °C for 5 min followed by chilling on ice for 5 min. The glucose formed was determined using a Glucose Assay Kit according to the supplier's instruction (Sigma). One unit of neutral trehalase activity is the amount that hydrolyses 1µmole of trehalose in 1hr. Protein concentrations were measured as described by Bradford (1976).

2.14.8 Southern analysis of RT-PCR

Southern analysis of RT-PCR was performed according to the method described by Dallman *et al.* (1991). RT-PCR was carried out using the Reverse Transcription System

according to the manufacture's instruction (Promega). An aliquot of the mRNA prepared from 100µg total RNA was used as template of RT-PCR, the primers were RTF and RTR (Table 2.1). The amplification protocol was as followings: First, RT was at 48 °C for 45min, then a denaturation step at 94 °c for 2min followed by 20 cycles of the following steps: denaturation at 94 °C for 30s, annealing at 62 °C for 1min, extension at 72 °C for 2min. A last elongation step was carried out at 72 °C for 7 min. Amplification was limited to 20 cycles in order to remain in a linear range and therefore produce semi-quantitative data. The sense primer RTF is located in the upstream of the first intron and the antisense primer RTR is located in the downstream of the second intron in the neutral trehalase gene. Therefore, amplification from genomic DNA yields a 905bp product whereas amplification from reverse-transcribed mRNA yields a 792bp product. RT-PCR products were separated on a 1.5 % agarose gel, and analysed by Southern analysis using the PCR product amplified using the cloned cDNA as template with primers RTF and RTR as probe. Hybridization conditions were fairly stringent (65 °C), the final wash conditions were 0.01×SSC, 0.1% SDS at 65 °C.

Table 2.1 Oligonucleotides used in this study

Oligonucleotide	Sequence
NtreF1	5'-GTNCCNGGNGGNAGRTTYAAYGA-3'
NtreF2	5'-GTNCCNGGNGGNCGNTTYAAYGA-3'
NtreR	5'-CCANCCRTANGGRTARTCCCAYTG-3'
NtreCF	5'-TGATACATGGCGGGAACGAC-3'
NtreCR	5'-TCACGGCAAATCAGCCAATTC-3'
NtreEF	5'- GATTGGATCCATGGCGGGAACGAC-3'
NtreER	5'- ATTCACTCGAGATTCACGGCAAATCAG-3'
RTF	5'- GACTATAATGTGTTTGACGACGC-3'
RTR	5'- TCCAGGCACTACAAAGGGGAG-3'

CHAPTER THREE

ACID PHOSPHATASES IN THE HAEMOLYMPH OF THE DESERT LOCUST, *SCHISTOCERCA GREGARIA*, INFECTED WITH THE ENTOMOPATHOGENIC FUNGUS *METARHIZIUM ANISOPLIAE*

3.1 INTRODUCTION

Insect fungal pathogens invade their hosts through the external skeleton. A battery of extracellular cuticle degrading proteases and chitinases facilitate passage through the integument and provide nutrition for the fungus (Charnley and St Leger, 1991; St Leger, 1995). Once in the insect, Deuteromycete pathogens like *Metarhizium anisopliae* proliferate to a greater or lesser extent in the haemolymph, where they may be confined prior to host death. Efficient use of nutrients in the haemolymph while combating the insect's blood-borne defences will be critical to successful parasitism. Good evidence implicates pathogen hydrolases in penetration of the exoskeleton, but the role of extracellular fungal enzymes in haemolymph has received little attention. Cuticle degrading subtilisin fungal proteases such as PR1 have powerful general proteolytic activity (St Leger *et al*, 1986a). However, they are generally absent from the haemolymph due to repression by low molecular molecular weight compounds.

Simple sugars (principally the diasaccharide trehalose) and phosphorylated sugars (e.g. glucose-1-phosphate) are found at high concentration in the haemolymph. While

repressive for fungal proteases they are a readily available source of carbon for the fungus, though utilisation may depend on secretion of the appropriate hydrolase. The necessary enzymes, α -glucosidase/trehalase and acid phosphatase, are secreted in culture by *M. anisopliae* (St Leger *et al*, 1986b; Xia, Clarkson and Charnley, unpubl).

Attempts to identify fungal enzymes in insect haemolymph must take into account the fact that defense related proteins, including enzymes, may be produced by the host in response to the fungal invasion. The insect immune system comprises a battery of humoral and cellular defences that can interact in the destruction of pathogens (reviewed by Gillespie *et al.*, 1997). The first line of defense in the haemolymph is often phagocytosis. This is carried out primarily by the plasmatocytes. Co-operative attack by species of insect haemocytes can lead to immobilization of groups of pathogens within granulomas (nodules) and the encapsulation of larger invaders. So called humoral defences such as production of melanin and antimicrobial peptides also play their part.

The study of invertebrate haemocyte cell killing mechanisms is fragmentary; lysozyme has been well studied but there is little information on other hydrolases (Anderson and Cook, 1979; Zachary and Hoffmann, 1984). Recently there have been a number of reports on the cytochemistry of the blood of oysters and mussels (Alvarez *et al.*, 1995; Anderson *et al.*, 1992; Anderson, 1994). The few studies on insects have concentrated on the characterization of naïve haemolymph (Chain and Anderson, 1983; Miranpuri *et al.*, 1989). Phagocytosis is known to stimulate production of lysosomal enzymes of which acid phosphatase (AcP) is a key component. AcP has been found in insect haemocytes and shown to be released into the plasma (Lai-Fook, 1973; Rowley and Ratcliffe, 1979).

Cheng (1983) reported hypersynthesis of acid phosphatase by haemocytes of the mollusc *Biomphalaria glabrata* during phagocytosis. The enzyme was subsequently released into the plasma where its role is unknown though alteration of surface properties of foreign particles by the enzyme may aid immunological recognition though a direct role of AcP in cell killing can not be ruled out.

We showed recently that mycosis of the desert locust with *M. anisopliae* var *acridum* resulted in changes in the properties of the haemolymph that occurred in two stages (Gillespie *et al*, 1999). During the first stage, in the first 2 days after inoculation, there was an increase in total haemocyte count (which was due in large part to an increase in coagulocytes), the number of nodules and prophenoloxisase (pPO) activity. All of these parameters were shown to change with respect to the control values when there was no or only a minor presence of fungus in the haemolymph. This suggests that there is a “signal” which is either a host derived molecule (released from the integument during fungal penetration) or a soluble fungal metabolite that activates the immune system. It is apparent that, whilst the immune system is stimulated by the fungus, the impact of the cellular host defenses on *M. anisopliae* var *acridum* is minimal since the haemocytes remain unattached to fungal particles and there is no indication that nodules incorporate fungus.

The second stage of the infection process occurred when the fungus had entered the haemocoel and replicated extensively (3-4 days after inoculation). At this time, all parameters measured (apart from pPO) were at levels significantly below those of controls and mycosed locusts in stage 1 of infection. This may be because the immune system has now been overcome by the fungus or fungally-derived metabolites.

The object of the present study was to determine the effect of fungal infection on haemolymph acid phosphatase in the desert locust. It was hoped to be able to distinguish any effect of infection on host derived acid phosphatase, which would be part of the immune response, and enzyme secreted by the fungus to promote host invasion

3.2 RESULTS

3.2.1 Acid phosphatase activity in haemolymph of *S. gregaria* during infection with *M. anisopliae* var *acridum*

Acid phosphatase activity was determined in whole haemolymph of *S. gregaria* during infection with *M. anisopliae* var *acridum* (Table 3.1). Three and four days after inoculation there were no significant differences in AcP activity between experimentals and controls. However, there was a large significant increase in AcP activity in mycosed insects on day 5 that was accentuated on day 6. The situation in fungus-infected insects may be contrasted with that in insects that were wounded (injected with saline) or treated in such a way as to stimulate directly the immune system (laminarin injection) (Table 3.2). Both treatments caused a significant increase in AcP 1d after injection, with further enhancement on day 2. In the next experiment the AcP activity was determined in the haemocyte and plasma fractions separately (Table 3.3). In the controls there was no significant difference in activity between the haemocytes and plasma. Furthermore the activity in the controls did not change significantly over the 3d of the experiment. However, there was a large, significant increase in haemocyte AcP activity following both

saline and laminarin injection. The activity in the haemocytes was significantly greater than in the plasma for both treatments on all three days.

Table 3.1 The effect of topical application of 1×10^4 conidiospores of *M. anisopliae* var *acridum* on haemolymph acid phosphatase activity in adult male *S. gregaria*

Day after infection	Acid phosphatase activity ($\mu\text{M pNP}/100\mu\text{l}$ haemolymph/h) ^a	
	control ^b	infected
3	0.462 ± 0.03^a	0.378 ± 0.08^a
4	0.432 ± 0.03^a	0.325 ± 0.03^a
5	0.349 ± 0.05^a	1.437 ± 0.18^c
6	0.424 ± 0.04^a	1.783 ± 0.12^c

^a Control insects that were inoculated with cotton seed oil and maintained in conditions identical to infected insects.

^b Mean \pm standard error, N = 9.

Means within a column or across a row followed by different superscript letters are significantly different at $p \leq 0.05$

Table 3.2 Acid phosphatase activity in whole haemolymph of *S. gregaria* following injection with saline or laminarin

Acid phosphatase activity ($\mu\text{mol}/100\mu\text{l}$ haemolymph/h)			
days-post injection	Uninjected control (n = 6)	Saline injected (n = 12)	Laminarin injected (n = 12)
1	0.41 ± 0.05^a	0.63 ± 0.049^b	0.56 ± 0.037^b
2	0.39 ± 0.03^a	1.02 ± 0.091^c	1.07 ± 0.081^c
3	0.45 ± 0.04^a	1.01 ± 0.009^c	1.18 ± 0.11^c

Means within a column or across a row followed by different superscript letters are significantly different at $p \leq 0.05$

Table 3.3 Acid phosphatase activity in components of the haemolymph of *S. gregaria* following injection with saline or laminarin

Acid phosphatase activity ($\mu\text{mol}/50\mu\text{l}$ haemolymph/h)						
Days- post injection	Uninjected control (n = 6)		Saline injected (n = 12)		Laminarin injected (n = 12)	
	Haemocytes	Plasma	Haemocytes	Plasma	Haemocytes	Plasma
1	0.387 $\pm 0.08^a$	0.254 $\pm 0.03^a$	0.497 $\pm 0.12^a$	0.227 $\pm 0.023^c$	0.554 $\pm 0.102^a$	0.238 $\pm 0.051^c$
2	0.444 $\pm 0.13^a$	0.271 $\pm 0.02^a$	1.062 $\pm 0.10^b$	0.321 $\pm 0.041^c$	1.326 $\pm 0.417^b$	0.358 $\pm 0.034^c$
3	0.450 $\pm 0.12^a$	0.291 $\pm 0.01^a$	1.019 $\pm 0.15^b$	0.359 $\pm 0.034^c$	1.180 $\pm 0.09^b$	0.356 $\pm 0.044^c$

For a given treatment, means within a column or across a row followed by different superscript letters are significantly different at $p \leq 0.05$

3.2.2 The impact of mycosis on acid phosphatase activity in the three classes of haemocyte of *S. gregaria*

Haemocyte monolayers were used to explore the effect of the treatments on AcP activity in individual sub-populations of haemocytes. The cells that showed intense dark red staining were characterised as AcP positive, some cells were a diffuse red colour or had no staining and were deemed AcP negative (Figure 3.1a, 3.1b).

Table 3.4 shows the impact of mycosis on AcP activity in the three classes of haemocyte. Whilst the proportion of stained granular cells (GRs) remained steady after inoculation, the proportion of AcP positive plasmatocytes (PLs) was initially less than controls, then increased briefly back to the control level (day 2). Conversely, the proportion of AcP positive coagulocytes (COs) steadily decreased. The experiment could not be continued up to day 4 post inoculation as haemocytes from insects at this stage of infection failed to adhere in significant numbers to the glass slide. The increase in the proportion of PLs in infected blood staining for acid phosphatase activity may relate to nodule formation in which PLs are extensively involved, consistent with this Figure 3.1e shows a nodule that stains intensely for AcP.

Elements of the fungus (hyphal bodies) observed in the blood on days 3 and 4 stained for acid phosphatase activity also (Figure 3.1f). This indicates that at least some of the AcP activity in infected blood may come from the fungus rather than the insect.

If monolayers of naïve haemolymph were prepared and overlaid with a 1 mg/ml solution of zymosan or laminarin (β ,1-3-glucans) for 1 hour prior to staining for AcP, there was a change in the differential staining pattern (Table 3.5). The proportion of COs and GRs that were acid phosphatase positive decreased with both zymosan and laminarin. The PLs were only affected by laminarin. If monolayers were incubated with *in vitro* produced blastospores or mycelial fragments, there was no change in the number of cells that stained for acid phosphatase activity (data not shown).

This research has shown a large increase in AcP activity in the haemolymph of insects injected with saline or laminarin and also of insects infected with *M. anisopliae* var *acridum*. In the case of the injury and chemically induced increase in AcP, the enzyme was largely localized in the haemocytes.

Figure 3.1 Photomicrographs of monolayers of haemocytes from *S. gregaria* and hyphal bodies of *M. anisopliae* var *acridum* stained for acid phosphatase:

- (A) plasmatocytes (a) and granular cell (b); scale bar= 25 μm ;
- (B) coagulocytes showing intense (a), diffuse (b) and non-staining (c); scale bar= 17.5 μm
- (C) plasmatocyte showing large acid phosphatase rich vesicle after incubation with zymosan; scale bar= 10 μm
- (D) diffuse staining coagulocyte undergoing "blebbing" with migration of acid phosphatase-rich granules to the periphery; scale bar= 10 μm ;
- (E) aggregation of haemocytes (nodule) on a monolayer stimulated with mycelial fragments; scale bar= 25 μm ;
- (F) hyphal bodies of *M. anisopliae* var *acridum* from the blood of a locust 4 days post inoculation. The hyphae stained positive for acid phosphatase; scale bar= 25 μm .

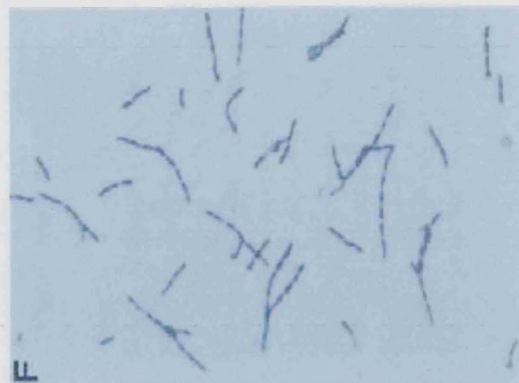
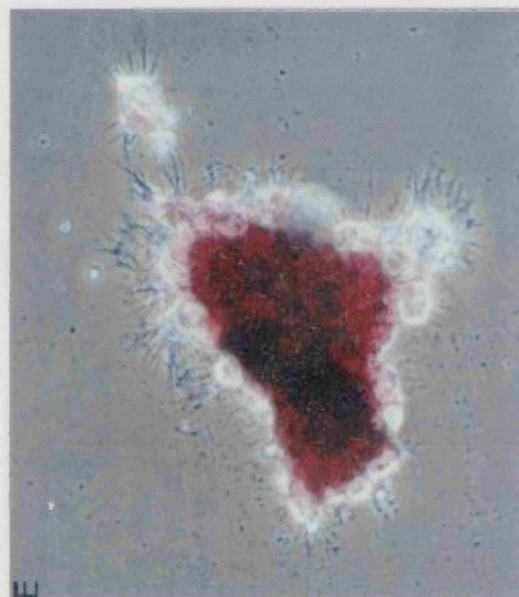
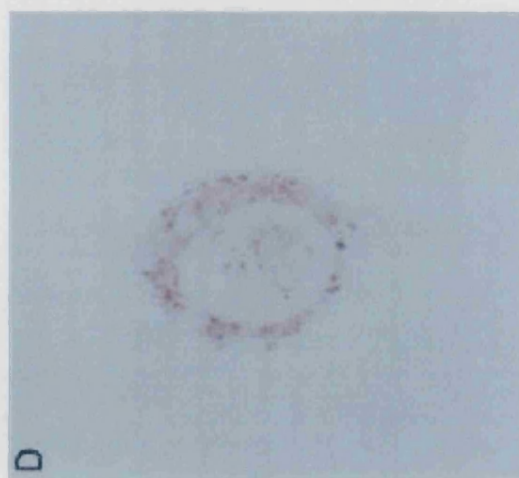
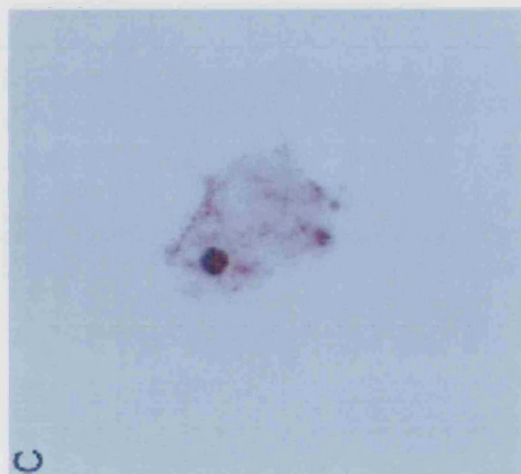
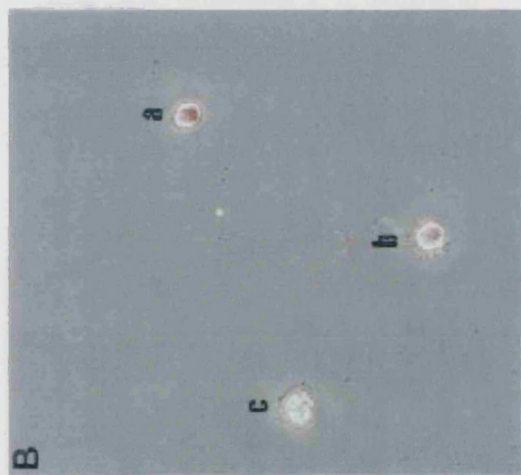
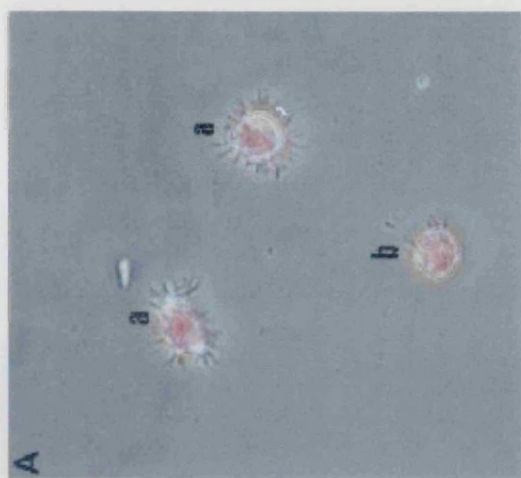


Table 3.4 The effect of topical application of 7.5×10^4 conidiospores of *M. anisopliae* var *acridum* on haemocytes that stained positively^a for acid phosphatase activity in adult male *S. gregaria*.

Day after inoculation ^b	% Staining Cells \pm SE, n = 12		
	CO	PL	GR
Control	88 \pm 5	58 \pm 3	100 \pm 0.00
1	60 \pm 5 *	40 \pm 4 *	84 \pm 12
2	50 \pm 4 *	75 \pm 11	100 \pm 0.00
3	15 \pm 5 *	8 \pm 8 *	100 \pm 0.00
4	N.A. ^c	N.A. ^c	N.A. ^c

^a Control insects were inoculated with cotton seed oil and maintained in conditions identical to infected insects and sampled at the same time points, the results were combined.

^b Positive cells were intense staining. A total of 200 cells were counted per monolayer,

^c Not attempted as there were less than 100 cells per monolayer, * Significantly different from corresponding control value ($p < 0.05$).

Table 3.5 The effect of β -1,3-glucans on acid phosphatase staining of haemocytes

Overlay	% intense staining cells ^{a,b}		
	CO	PL	GR
None	81.25 \pm 1.10	61.75 \pm 1.31	100.00 \pm 0.00
Zymosan	33.25 \pm 1.38 *	61.75 \pm 1.65	73.5 \pm 0.75 *
Laminarin	38.00 \pm 1.47 *	55.00 \pm 1.29 *	64.75 \pm 1.55 *

^a Figures reported plus or minus standard error. n=12.

^b Positive cells were intense staining cells; 200 cells were counted per monolayer

* Significantly different than corresponding control value (p<0.05)

3.2.3 Acid phosphatase isoforms in the plasma of *S. gregaria* during infection with *M. anisopliae*

IEF of plasma from locusts of different treatments resolved AcP into a number of isoforms. Control (oil treated) locusts had just one AcP with a pI of 4.3 (see Fig 3. 2). Injection with laminarin resulted in increased activity of the 4.3 enzyme and the appearance of 3 new isoforms between pIs 7.3 and 7.5. Locusts infected with *M. anisopliae* var *acridum* did not exhibit the changes seen upon injection with laminarin, instead 2 new isoforms appeared at pI 7.8 and 8.0. These enzymes correspond to AcP isoforms produced in culture by *M. anisopliae* var *acridum* and var *anisopliae*(Fig3.2). Locusts infected with var *anisopliae* showed increased activity at pI 4.3 in the early stages of infection (2d and 4d) which was much reduced by day 6. No other isoforms were identified.

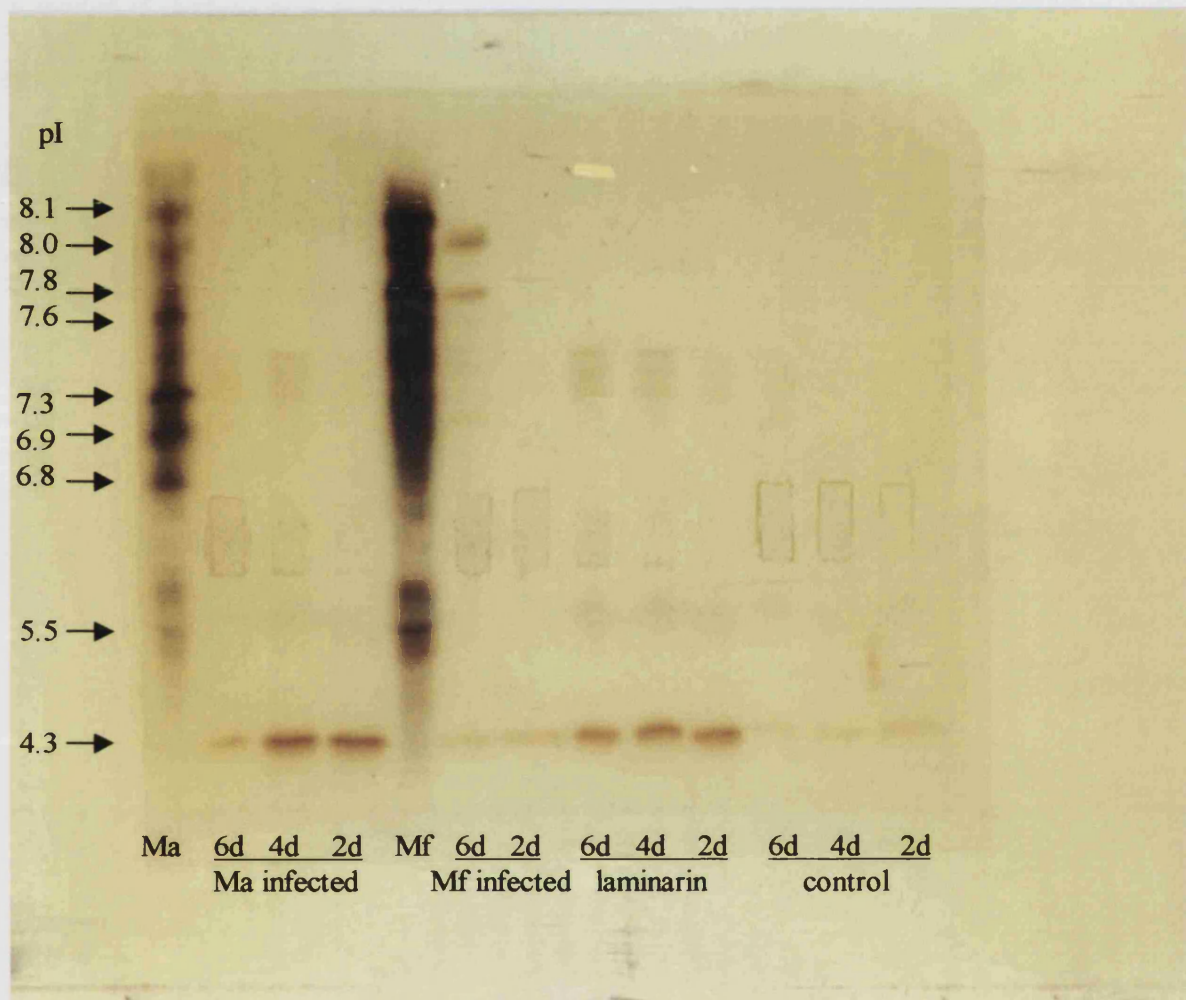


Figure 3.2. Isoelectric focussing gel of haemolymph plasma from the desert locust, *Schistocerca gregaria* and supernatant from *in vitro* fungal cultures stained for acid phosphatase activity.

Legend:

Ma = isoforms separated from the supernatant of an *in vitro* culture of *M. anisopliae* var *anisopliae*

Ma infected = plasma taken from locusts at 2, 4 and 6d after inoculation with *M. anisopliae* var *anisopliae*

Mf = isoforms separated from the supernatant of an *in vitro* culture of *M. anisopliae* var *acridum*

Mf infected = plasma taken from locusts at 2 and 6d after inoculation with *M. anisopliae* var *acridum*

laminarin = plasma taken from locusts 2, 4 and 6d after injected with laminarin

control = plasma taken from locusts 2, 4 and 6d after topical application with 2μl of oil (the carrier for both fungal treatments).

3.3 DISCUSSION

Untreated control locusts had constitutive levels of AcP in their haemolymph. As a lysosomal enzyme AcP may have a role in autophagy and cell turn over as well as defence. Wounding caused by saline injection caused a significant increase in haemolymph AcP. The increase was greater in the haemocytes than the plasma suggesting that the enzyme derives from the cells. Degranulation of coagulocytes and release of lysosomal enzymes was observed during coagulation at a wound site in *Locusta migratoria* (Zachary and Hoffman, 1984). Increase in the proportion of phagocytically competent cells with injury (Yeaton, 1983) may account for the enhanced haemocyte AcP activity. It is interesting that in the present work although an initial increase in AcP activity occurred within a day of treatment a more pronounced effect occurred several days after saline injection. The solutions were all made up in sterile, endotoxin-free water and there was no evidence of bacterial septicaemia. Therefore elevated AcP appears to be a long term effect of the needle wound and small saline infusion. Gunnarsson and Lackie (1985) showed enhanced nodulation, phagocytosis and haemocyte degranulation in *S. gregaria* following injection of β -1,3-glucans (including laminarin). In the present work stimulation of the immune system by laminarin was also suggested by a dose dependent increase in melanin production in the haemolymph (data not shown). However, AcP activity was not significantly greater in β -1,3-glucan treated insects than in those injected with saline alone. It is not clear why the stimulatory effects of laminarin on the immune system did not extend to AcP activity. It may be that the depletion of the circulating pool of haemocytes due to participation in nodule formation counterbalances AcP induction in individual cells.

Furthermore at least *in vitro*, on haemocyte monolayers, laminarin and zymosan decrease the proportion of cells that stain for AcP activity (see following discussion).

AcP activity also increased in the haemolymph on the 3rd day after inoculation with the entomopathogenic fungus *M. anisopliae* var *acridum*. This is 2 days later than the initial increase in AcP following injection with saline or laminarin. On the 3rd day of mycosis the total haemocyte count (THC) after an initial increase started to decline (Gillespie *et al*, 1999) and there was a marked reduction in the proportion of PLs and COs that stain positive for AcP (Table 4). Therefore *a priori* it seems unlikely that the extra AcP in infected insects comes from the host. Overlays of β -1,3-glucans on haemocyte monolayers created from naive haemolymph significantly reduced the proportion of COs, GRs and PLs (laminarin only) that stained for AcP. Chemical stimulation of the haemocytes may cause release of AcP. The results of the *in vitro* study in part correspond to the *in vivo* work. In both there is a reduction in the proportion of COs and PLs that stain positive for AcP. The exceptions are the GRs whose AcP staining properties are not affected by fungal infection but are by exposure to β -1,3-glucan. These results lend substance to the view that the effects of fungal infection on the AcP activity of haemocytes are a combination of the stimulatory effects of non-self and the detrimental effects of the fungus.

Control inoculated (oil only) insects have an AcP with a pI of 4.3 that, judging by band intensity on an IEF gel, is further stimulated by the injection of laminarin. Additional isoforms appear at around 7.3-7.5 in the laminarin treatment. However, the 4.3 isoform appears to be suppressed in the insects infected with *M. anisopliae* var *acridum*. The band

intensity is more like that of the control than the laminarin-injected insects. The suppression of host AcP, or failure to up-regulate because of a decline in the quantity and quality of the haemocytes, is consistent with the measurements of AcP activity in the haemolymph. Wounding and laminarin both caused an increase in whole haemolymph AcP within a day of treatment. In contrast enhanced activity in mycosed haemolymph was delayed until 3 days post inoculation, when elements of the fungus were evident. Two new isoforms of AcP, noted in 6d haemolymph, may be responsible for elevated AcP in mycosed insects. A fungal origin for these isoforms is suggested by the fact that they have pIs that correspond to some of the AcPs produced *in vitro* by *M. anisopliae* var *acridum*. Intense AcP activity staining of fungal elements in haemolymph of locusts infected with *M. anisopliae* var *acridum* suggests that fungal enzyme may be bound to the cell wall as well as being secreted into the plasma.

Interestingly the picture is somewhat different in insects mycosed with an isolate of *M. anisopliae* var *anisopliae*. This fungus grows less profusely than *M. anisopliae* var *acridum* in host haemolymph prior to death (unpubl res), and appears only to suppress the host-derived 4.3 pI AcP later on in infection and appears not to secrete its own AcP.

It is not yet known why a fungus would secrete AcP into host haemolymph. However, sugar phosphates are found in high concentration in insect haemolymph (Wyatt, 1967). Hydrolysis of these compounds by the fungus would provide both phosphate and a source of energy. Interestingly, addition of fungal AcP to insect haemolymph caused decrease in glucose phosphate (Charnley *et al*, 1997) and a large significant increase in inorganic

phosphate concentration has been found in haemolymph from fungus infected insects (Xia *et al.*, unpubl). Alternatively fungal AcPs may target the immune system. Dephosphorylation of immune proteins that have been activated by phosphorylation may interfere with effective host defence. Significant qualitative and quantitative changes do occur in the pattern of phosphorylated proteins in host haemolymph during mycosis that could at least in part be caused by the activities of fungal AcP (Xia *et al.*, unpubl res).

The only other study on the effect of fungal infection on haemolymph AcP was that of Vincent *et al* (1993). They found that injections of a suspension of conidia of *Beauveria bassiana* in a solution of Tween 80 or Tween 80 alone into grasshoppers, *Melanoplus sanguinipes*, within 1-2h caused a large, brief increase in the percentage of haemocytes that stained for AcP. This appeared to be an injury response. A second more prolonged peak (at 48h) occurred only in the conidia injected insects presumably in response to the developing fungus. These observations were supported by quantitative measurements of AcP activity that differentiated cellular and plasma enzyme and suggested a haemocyte site of origin for AcP. Unfortunately they did not take into account a possible fungal contribution to AcP activity. It is difficult to make further comparisons with the present work because Vincent *et al* (1993) were looking at more short term effects. Furthermore unlike the present work where spores were applied topically, they injected their insects. This method of inoculation may not reveal the extent of the immune response since it bypasses the natural route of infection and involves a form of the fungus which is not normally present in the haemolymph of infected insects.

CHAPTER FOUR

ACID PHOSPHATASES OF *METARHIZIUM ANISOPLIAE* AND THEIR ROLE IN PATHOGENESIS OF THE TOBACCO HORNWORM, *MANDUCA SEXTA*

4.1 Introduction

Phosphorus is essential to the growth and well being of living things; insects and their fungal pathogens are no exception. Organic phosphates such as glucose-1-phosphate and trehalose-6-phosphate comprise a major proportion of the total phosphorus in the haemolymph of a number of lepidopteran spp. including *M. sexta* (Pannabecker *et al.*, 1992; Wyatt, 1961). Since insect pathogenic Deuteromycotina like *Metarhizium anisopliae* develop in the haemolymph after host invasion, organic phosphates may be an important nutrient source for fungal growth. Efficient utilisation of phosphorylated organic molecules requires production of phosphatases, which hydrolyse phosphate (Pi) from orthophosphate monoesters.

All phosphatases catalyse the same biological reaction but may be grouped according to their pH optima viz acid (AcP) and alkaline phosphatases (Wynne & Bergstein Bendan, 1995). Alkaline phosphatases generally display an absolute substrate specificity, AcPs do not.

Total AcP activity increases dramatically in the haemolymph of grasshoppers infected with the fungus *Beauveria bassania* (Vincent *et al.*, 1993). Hypersynthesis of this enzyme also occurs after bacterial infection in mammals (Cheng & Butler, 1979). In both cases increased phosphatase was attributed to the host immune response. However, entomopathogenic fungi also secrete AcP in culture (St Leger *et al.*, 1986; St Leger *et al.*, 1992; Bridge *et al.*, 1993), so the increase of AcP activity after fungal invasion may, at least in part, be due to the fungus.

Good evidence implicates pathogen hydrolases in penetration of host exoskeleton, but the role of extracellular fungal enzymes in haemolymph has received little attention. We describe here the purification and partial characterisation of the main AcP isozymes produced *in vitro* by *M. anisopliae* isolate ME1, and present evidence that fungal AcPs are secreted in the haemolymph of mycosed larvae of the tobacco hornworm, *Manduca sexta*.

4.2 Results

4.2.1 Regulation of acid phosphatase *in vitro*

A series of experiments with variations of inorganic phosphate, pH of culture, inoculation size and age of culture were done in liquid culture to investigate the regulation of acid phosphatase from *M. anisopliae* strain ME1. Regulation of AcP was studied to optimise the culture conditions for AcP purification in term of specific activity and number of AcP isoforms. Fig. 4.1 shows the effects of inorganic phosphate (Pi) concentration on

the production of extracellular AcP in cultures of *M. anisopliae*. Activity on the fourth day was 3 fold greater in moderate Pi (10 mg Pi L⁻¹) than in high Pi (250 mg Pi L⁻¹), indicating induction by the former and repression by the latter. Five major AcP isozymes were produced in both regimes and at least 5 additional isoforms (pI8.0, 7.7, 7.6, 7.3, 6.9) were produced in the Pi-limited medium (10 mg Pi L⁻¹) (Fig. 4.2), suggesting differential regulation of isoforms.

The optimum conditions for AcP production (in terms of specific activity and number of isoforms) were determined as an inoculum of 10⁸ spores to 100ml medium (basal salts + 2 % glucose + 0.2 % NaNO₃ + 10mg Pi L⁻¹ at pH 6.0) in 250 ml flask, shaken at 150 rpm at 27 °C for 4 days.

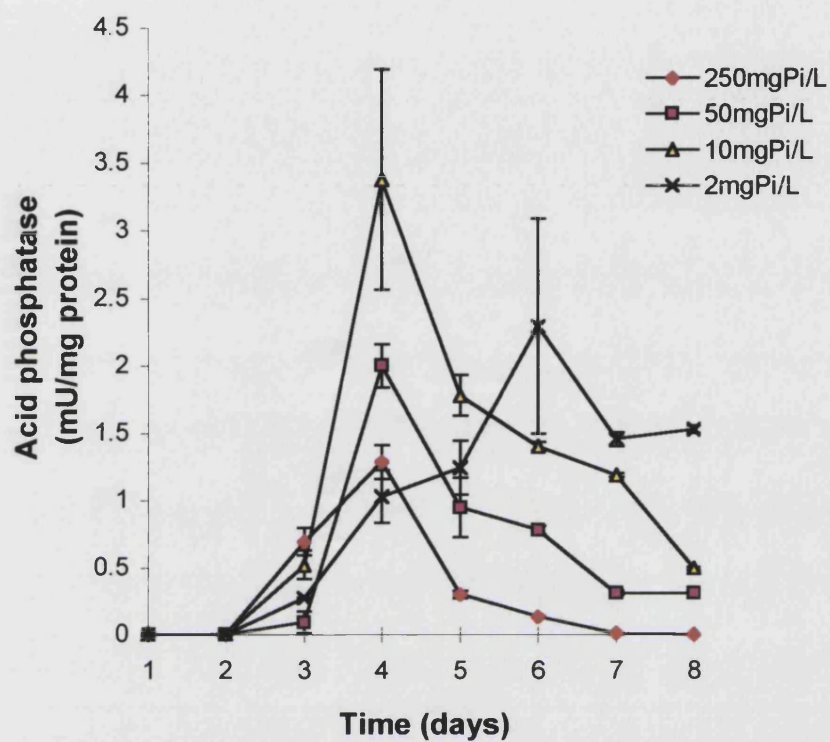


Fig 4.1 Effect of Pi concentrations on acid phosphatase production

1.0×10^7 ME1 spores were inoculated into 100 ml medium (pH6.0) containing different concentration of Pi concentrations (250, 50, 10, 2 mg Pi/L) in 250 ml flasks, $n=3$, mean \pm SD.

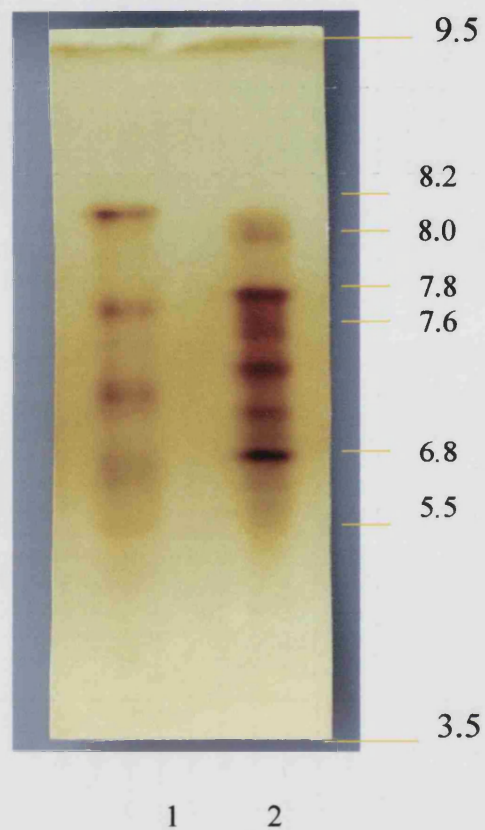


Fig 4.2 Acid phosphatase isozymes of *ME1* analysed by IEF-PAGE (3.5 - 9.5).

1, Full medium (250mg Pi/L): 30 μ g protein applied

2, Pi-limited medium (10mg Pi/L): 10 μ g protein applied

4.2.2 Purification and partial characterisation

AcPs were purified from filtrates of 4-day cultures of *M. anisopliae* grown under the optimised culture conditions using Con A - Sepharose and narrow range IEF. Three isoforms with pI values of 7.8, 8.0 and 8.1, were purified to homogeneity as indicated by silver staining of a SDS-PAGE gel (Fig. 4.3). The progress of each purification step is summarised in Table 4.1.

The activity of purified AcP isoforms was determined against sugar phosphates including some found in insect haemolymph. All substrates were hydrolysed by the purified fungal acid phosphatases, including the synthetic phosphate *p*NPP (*p*-nitrophenol phosphate). However, there were obvious differences among the three isoforms against sugar phosphates found in insect haemolymph. The isoform pI 8.1 was inactive against fructose-6-phosphate, but it was equally active against glycerol-3-phosphate and glucose-6-phosphate. The activity of isoform 8.0 against glycerol-3-phosphate was very low, but enzyme hydrolysed fructose-6-phosphate and glucose-6-phosphate. The activities of isoform pI7.8 against all the sugar phosphates tested were much higher than that of the isoforms 8.1 and 8.0. The activity of the isoform 7.8 against fructose-6-phosphate was more than 4 times higher than the isoform 8.0, the activity of the isoform 7.8 against glycerol-3-phosphate was 7 times higher than the isoform 8.1, the activity of the isoform 7.8 against glucose-6-phosphate was more than 5 times higher than the isoform 8.1 (Table

4.2). The isoform 7.8 was much more efficient in hydrolysing sugar phosphates found in haemolymph.

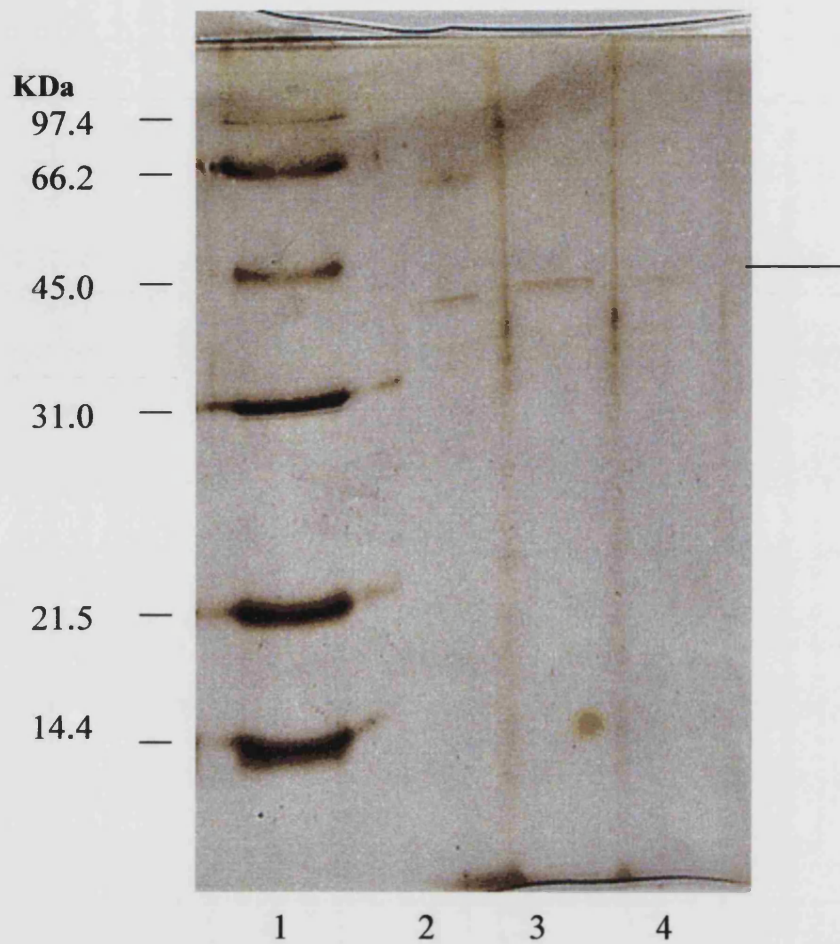


Fig 4.3 Purified Acid Phosphatase Isoforms On SDS-PAGE (15%)
With Silver-Staining

- 1: Molecular Markers; 2: Acid phosphatase with pI 8.1;
3: Acid phosphatase with pI 8.0; 4: Acid phosphatase with pI 7.8

Table 4.1 Purification of *M. anisopliae* acid phosphatase

Fraction	Total protein (mg)	Total activity (U)	Specific activity (mU/mg)	Purification Factor	Yield (%)
Filtrates of culture	693.8	2.536	3.7	----	----
ConA elute	72.5	2.391	33.0	8.9	94.3
IEF gel elute:					
pI 8.1 isoform	0.437	0.175	400.5	108.2	6.9
pI 8.0 isoform	0.222	0.098	441.4	119.3	3.9
pI 7.8 isoform	0.335	0.436	1301.5	351.8	17.2

Table 4.2 Substrate specificity of acid phosphatase isozymes

(U*, n=3, mean \pm SD)

Isoforms	Glucose-6-phosphate	Glycerol-3-phosphate	Fructose-6-phosphate	<i>p</i> -nitrophenol phosphate
PI 8.1 isoform	0.138 ± 0.010	0.124 ± 0.005	0.000 ± 0.001	0.183 ± 0.008
PI 8.0 isoform	0.104 ± 0.006	0.025 ± 0.021	0.101 ± 0.014	0.182 ± 0.011
PI 7.8 isoform	0.741 ± 0.020	0.858 ± 0.037	0.455 ± 0.018	0.754 ± 0.013

*1U = 1 μ M phosphate released min⁻¹.mg protein⁻¹

4.2.3 Acid phosphatase in the haemolymph of infected *Manduca sexta*

AcP activity in cell-free haemolymph was measured using *p*-nitrophenylphosphate (p-NPP) (Wynne 1995). Activity in haemolymph from infected insects (live spore treated) increased ca two fold and was significantly greater than in healthy insects or either set of controls (dipped in dead spores or 0.05 % Tween 80) at 5 days after treatment (Fig. 4.4). However, dead spores caused a significant elevation of enzyme activity ($p < 0.01$) over that in healthy insects.

AcP isoforms were separated on flat IEF gels and visualised using naphthyl phosphate as substrate. No AcP isoforms were detected in the haemolymph of infected 5th instar *M. sexta* larvae using this substrate until 60h post inoculation. At this time two AcP isoforms appeared; a further isoform was detected on day 6. The three isozymes (pI 7.1, 7.3, 8.1) had similar pI values to some of the AcP isozymes in fungal culture (Fig. 4.5). In contrast to the situation in haemolymph from mycosed larvae, although there was relatively high AcP activity in control haemolymph only one weak band with pI 7.1 was visualised. This appears to be because AcPs of *M. sexta* degraded naphthyl phosphate much less effectively than did the fungal enzymes.

The strong band with pI 7.1 on IEF of haemolymph from infected larvae 6 days post inoculation corresponded to a weak band of similar pI in the control insects as well as a major isoform in the fungal culture. The prominent pI 7.1 band in infected haemolymph

could therefore be caused by upregulation of an insect enzyme or secretion of a fungal enzyme. The other two AcP isoforms (pI 7.3 and 8.1) in infected haemolymph appear to be fungally derived. Isoforms with these pIs were detected in fungal culture but not in haemolymph from control or indeed non-treated insects from any stage of development examined (larva, pupa or adult) (data not shown).

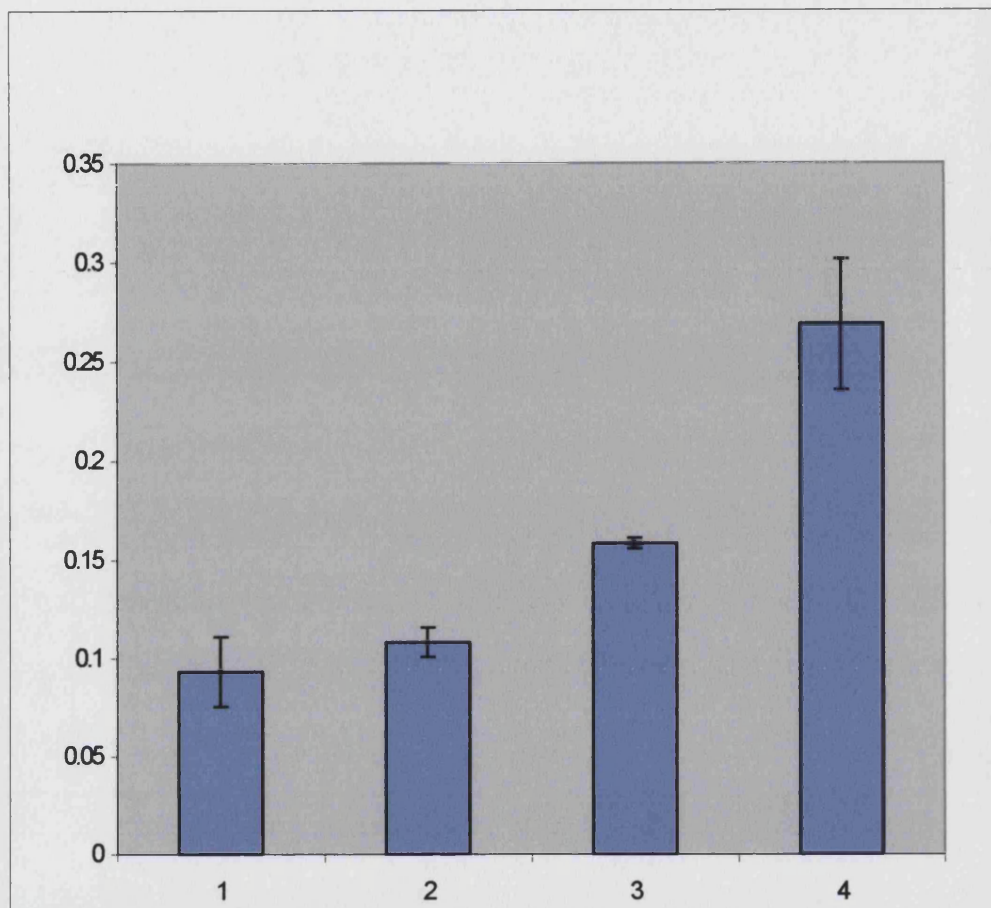


Fig 4.4 Acid phosphatase activity in the haemolymph of *Manduca sexta* with *M. anisopliae*. (U, n=3, mean \pm SE; 5 days after inoculation)

1U= $1\mu\text{M}$ phosphate released min^{-1} /ml haemolymph

1, Healthy

2, Dipped in 0.05% Tween 80

3, Dipped in 1.0×10^6 dead spore suspension

4, Dipped in 1.0×10^6 live spore suspension

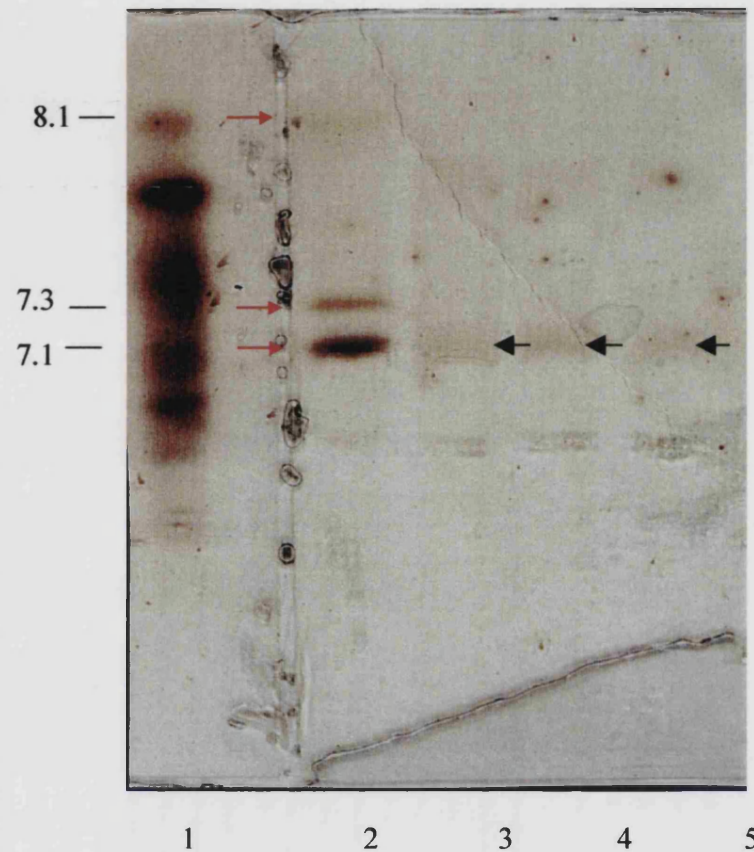


Fig 4.5 Acid phosphatase isozymes separated on IEF gel, from the haemolymph of *Manduca sexta* with *M. anisopliae* (6 days after inoculation)

1: ME1 culture filtrate

2: haemolymph of *Manduca sexta* dipped in 1×10^6 live spores (—)

3: haemolymph of *Manduca sexta* dipped in 1×10^6 dead spores (—)

4: haemolymph of *Manduca sexta* dipped in 0.05% tween 80 (—)

5: haemolymph of untreated control (—)

Further evidence for a fungal origin of, at least a proportion, the AcP activity in haemolymph of mycosed insects comes from experiments with the AcP antibodies. Antibodies were raised against the three purified isoforms of *M. anisopliae* AcP. The specificity of the antibodies was tested using AcPs from different sources. The results indicated that the antibodies were of high titre against target *M. anisopliae* AcPs (Fig. 4.6). Weaker cross-reaction occurred (at higher enzyme concentrations) with AcPs from human, plant and entomopathogenic fungi from other genera (Fig. 4.6). No cross-reactivity was found in dot - blots between anti - AcP IgG and haemolymph from healthy insects or from insects dipped in dead spores. Furthermore there was no cross - reaction with haemolymph from insects injected with laminarin (Fig. 4.7). This β - 1,3 glucan is a known stimulant of the insect immune response. However, a strong cross-reaction occurred between the antibody and haemolymph from 6d mycosed insects.

Western blot analysis of SDS - PAGE of haemolymph proteins identified a band in infected haemolymph with a similar MW to culture-derived AcP from *M. anisopliae* (Fig 4.8).

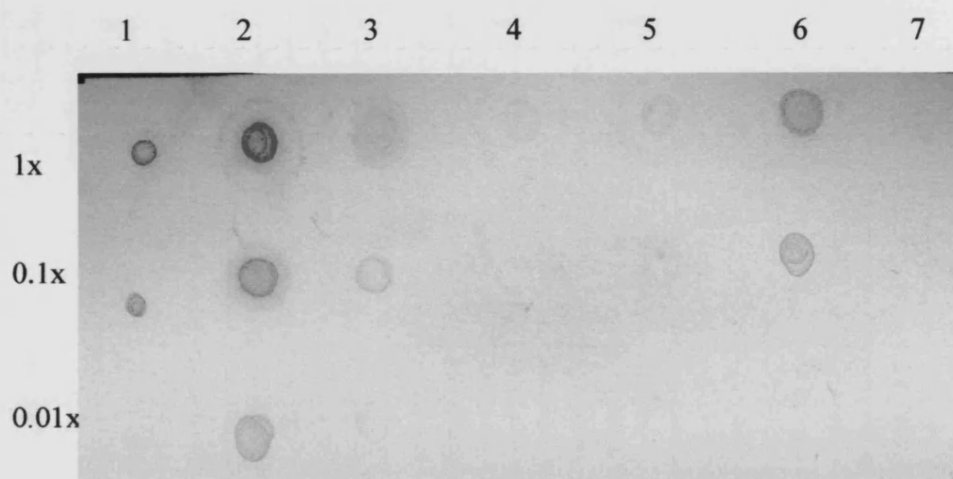


Fig 4.6 Specificity test of anti -acid phosphatase antibodies
by dot – blotting (dilution of antibodies: 1: 2000)

- 1: Purified *Metarhizium anisopliae* acid phosphatase (1X: 0.07 mg/ml)
- 2: *Metarhizium anisopliae* culture filtrate (1X: 2.93 mg/ml, 0.189U)
- 3: *Metarhizium flavoviride* culture filtrate (1X: 2.75 mg/ml, 0.038U)
- 4: *Verticillium lecanii* culture filtrate (1X: 3.92 mg/ml, 0.035U)
- 5: *Beauveria bassiana* culture filtrate (1X: 3.93 mg/ml, 0.041U)
- 6: Wheat germ acid phosphatase (1X: 1.23 mg/ml, 0.049U)
- 7: Human prostate gland acid phosphatase (1X: 0.26 mg/ml, 0.052U)

1 2 3 4

Fig 4.7 Cross - reactivity of acid phosphatase antibodies with
haemolymph from *M. sexta*

- 1: Haemolymph from live - spore treated *M. sexta* (6d after treatment)
- 2: Haemolymph from dead - spore treated *M. sexta* (6d after treatment)
- 3: Haemolymph from *M. sexta* injected with laminarin (6d after treatment)
- 4: Haemolymph from healthy *M. sexta* (6d after treatment)



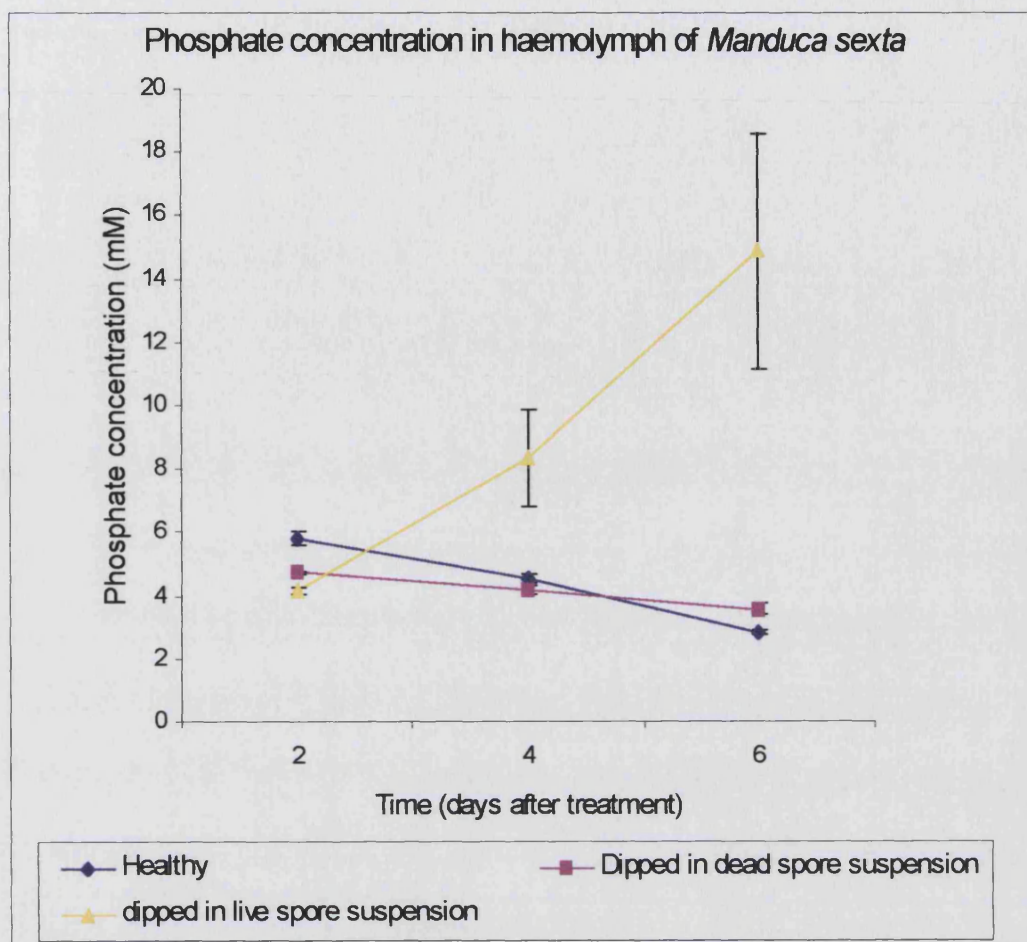
Fig 4.8 Detection of fungal acid phosphatase in the haemolymph of infected *Manduca sexta* by western blot from SDS -PAGE (15%) using anti - acid Phosphatase antibody (1:4000)

- 1: Haemolymph of healthy *M. sexta* (6d)
- 2: Haemolymph of dead - spore treated *M. sexta* (6d)
- 3: Haemolymph of live - spore treated *M. sexta* (3d)
- 4: Haemolymph of live - spore treated *M. sexta* (6d)
- 5: 25µl ME 1 culture filtrate; 6: 2.5µl ME1 culture filtrate

4.2.4 Phosphate concentration in the haemolymph of ME1 infected *M. sexta*

The phosphate concentration in the haemolymph of both sets of controls viz untreated healthy larvae and dead spore treated larvae, dropped steadily and significantly during development. However, phosphate concentration in the mycosed larvae increased dramatically once the fungus got into the haemolymph (3-4d). The phosphate concentration in the infected larvae was about 2 times that of both control treatments 4 days post inoculation and was about 5 times that of the two sets of controls 6 days after inoculation (Fig. 4.9).

Fig 4. 9 Phosphate concentration in haemolymph of *M. sexta* (n = 10, mean \pm SD)



First day 5th instar *M. sexta* larvae were dipped in $3 \times 10^6 \text{ ml}^{-1}$ live spore suspension or $3 \times 10^6 \text{ ml}^{-1}$ dead spore suspension. Phosphate (PO_4^{3+}) concentration was determined in cell free haemolymph samples.

4.2.5 Growth of *Metarhizium anisopliae* in media with different concentrations of phosphate

Fungal growth in a basal medium increased with phosphate concentration and peaked at ca. 16 mM (Fig. 4.10). Phosphate concentration in haemolymph of normal, non-infected 5th instar *Manduca* larvae was 4.5 mM (Fig 4.9). Fungal growth was significantly greater in cell-free haemolymph of four-day old 5th *Manduca* larvae augmented with exogenous phosphate and peaked at ca. 15 mM (Fig. 4.11), suggesting phosphate is a limiting factor for ME1 growth in the in cell-free haemolymph of four-day old 5th *Manduca* larvae.

Fig 4.10 Effect of Phosphate concentration on ME1 Growth in vitro
(n=3, mean \pm SD)

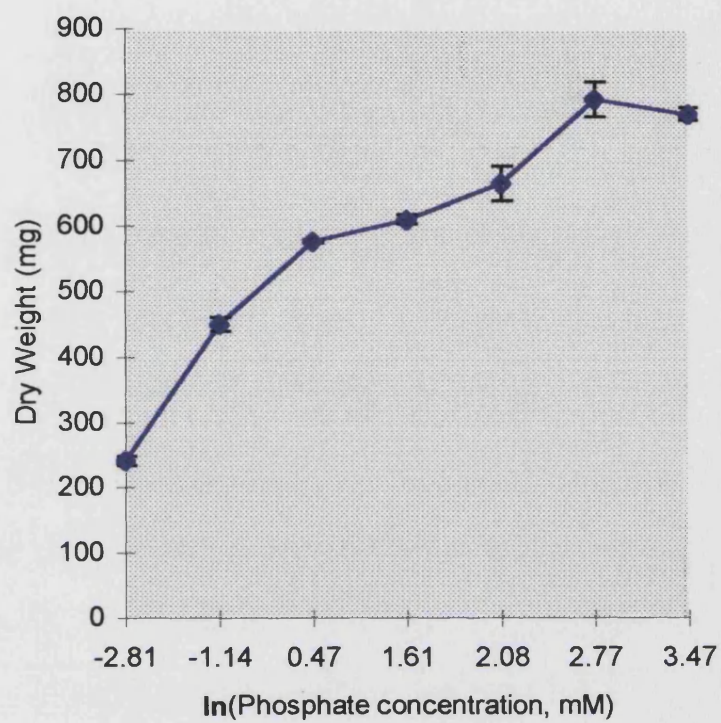
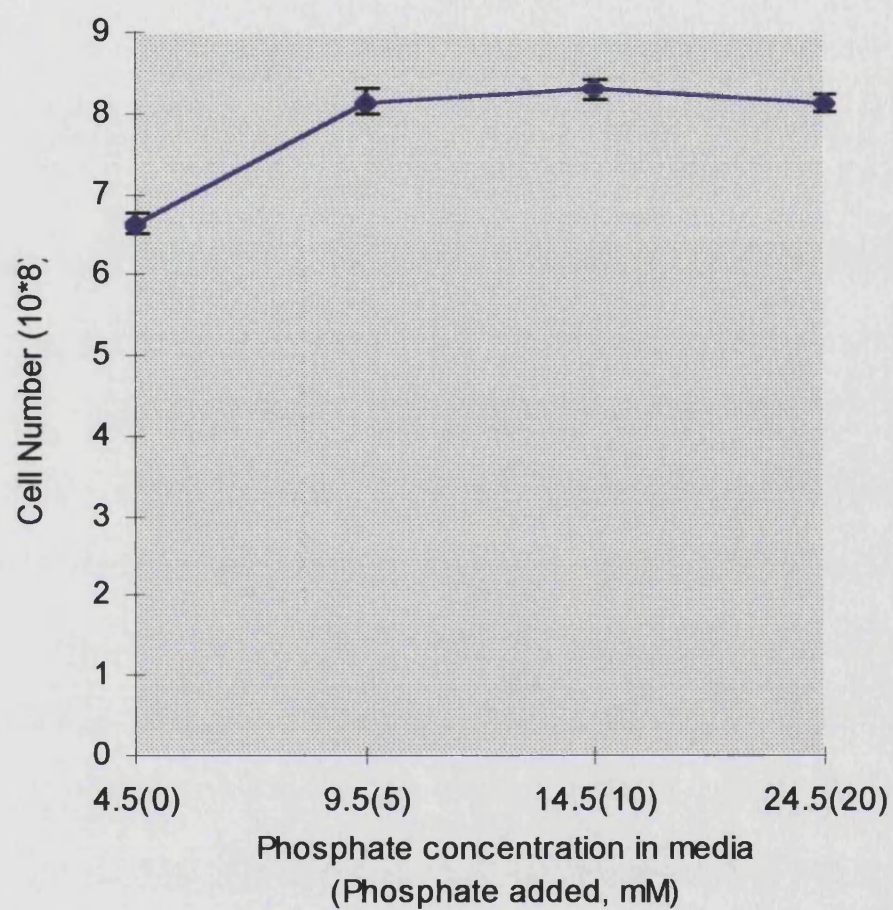


Fig 4.11 Effect of phosphate on ME1 growth in cell-free haemolymph
(n=3, mean \pm SD)



4.3 Discussion

AcPs of *M. anisopliae* are repressed by high concentrations of inorganic phosphate. In common with Pi-repressible AcPs in other filamentous fungi e.g. *Aspergillus niger* (Ryu & Lee, 1977; Ullah & Cummins, 1987; MacRae *et al.*, 1988), those in *M. anisopliae* are probably transcriptionally regulated by phosphate.

M. anisopliae ME1 produced at least 8 AcP isoforms with pIs that ranged from 5.5 - 8.1 under optimum culture conditions. Three basic AcP isozymes, pI 7.8, 8.1, and 8.0, were purified; they had similar molecular weights (ca.44.0 kDa).

AcP activity in haemolymph of infected *M sexta* larvae increased significantly. Vincent *et al* (1993) noted a similar phenomenon in grasshoppers infected with *B. bassiana* and attributed it to the immune response of the insect. However, present results with *Metarhizium* are consistent with a fungal origin for at least a part of the increase in AcP activity in mycosed *Manduca*. AcP of infected *M. sexta* was significantly greater than that of control treatments, and some extra basic AcP isoforms (pI 7.3 and 8.1) were detected in the haemolymph of infected larvae but not in uninfected controls. The prominent pI 7.1 isoform in mycosed haemolymph corresponded to a weak band in both control treatments. The significant increase in AcP activity, probably an immune response, caused by treatment with dead spores was not therefore expressed as an increase in the pI 7.1 band. Thus it seems highly likely that the large pI 7.1 band on IEF gels of mycosed haemolymph is caused a fungal enzyme rather than up regulation of insect-derived immune-related AcP.

Dot - blot and Western blot analyses using antibody raised against *in vitro* fungal AcP indicated the presence of fungal enzyme in haemolymph of mycosed *M. sexta*. Antibodies raised against pure *in vitro* fungal AcPs did not cross - react with immune (chemically stimulated) or non - immune haemolymph from *M. sexta*. However, a protein with similar MW (44 kDa) to the *in vitro* fungal AcP, present in haemolymph from mycosed insects, cross-reacted with AcP antibody at high dilution (1:1000).

As stated earlier the significant increase in AcP in dead spore treated insects is probably an immune response as AcP has been implicated in insect cellular defense (see review by Gillespie *et al.*, 1997). This is particularly interesting because the insects were merely dipped in a suspension of autoclaved spores, indicating the possibility of signal exchange between the surface of the cuticle and the haemolymph.

We have shown elsewhere that fungal AcPs may also be secreted by *M. anisopliae* var *acridum* during mycosis in the desert locust, *Schistocerca gregaria* (Xia *et al.*, 2000). In this case the fungus appears to be suppressing the host's own AcPs. Apparent failure of *Manduca* AcPs to degrade naphthyl phosphate, the substrate used for locating AcP in IEF gels, prevented us from determining whether *M. anisopliae* var *anisopliae* has a similar effect on *Manduca* AcP.

AcP isozyme(s) secreted by entomopathogenic fungi during mycosis may play an important role in the interaction between pathogen and host. Purified *Metarhizium* AcP isozymes can degrade a number of the phosphorylated sugars found *in vitro* and the pH of

Manduca haemolymph (~ 6.5) is close to the optimum of the fungal enzymes. Glucose-phosphate in haemolymph of *Manduca* caterpillars infected with *M. anisopliae* decreased conspicuously (Cobb, Clarkson and Charnley, unpubl), while free phosphate concentration increased dramatically in the late stage of mycosis (present work). Taken together these observations suggest that *in vivo* fungal AcP degrade organic phosphates in the host. Since inorganic orthophosphate (Pi) is the only P source that can be directly taken up and metabolized by microorganisms (Wynne, 1995), secreted fungal AcPs may facilitate growth of the fungus in host haemolymph by providing an essential ion as well as a useful source of energy (carbohydrate). Certainly growth of *M. anisopliae* is limited at phosphate concentrations pertaining in the haemolymph. Furthermore increasing phosphate concentration in cell free haemolymph up to levels achieved during mycosis significantly increased growth of *Metarhizium*. The marked increase in free phosphate in *Manduca* haemolymph suggests that the activity of the enzyme outstrips the requirements of the fungus or the capacity of its Pi transport system.

CHAPTER FIVE

TREHALOSE-HYDROLYSING ENZYMES OF *METARHIZIUM ANISOPLIAE* AND THEIR ROLE IN PATHOGENESIS OF THE TOBACCO HORNWORM, *MANDUCA SEXTA*

5.1 Introduction

Entomopathogenic fungi invade their hosts through the external skeleton (cuticle). Particular attention has been paid to the extracellular enzymes involved in this process. Cuticle-degrading proteases and chitinases have been well characterised and a number of genes cloned and sequenced (Charnley and St Leger, 1991; Smithson *et al*, 1995; St Leger *et al*, 1992; Bogo *et al*, 1998). However, little is known about the interactions between insect and pathogen once the fungus has got into the haemolymph.

The haemolymph of lepidopteran hosts of *M. anisopliae*, including *M. sexta*, has a high concentration of soluble sugars (Racioppi and Dahlman, 1980); prominent among them is the disaccharide, trehalose. The last-named is also the major sugar utilised during flight (Becker *et. al.*, 1996). Clearly trehalose must be viewed as a potentially important nutrient source for pathogenic fungi like *M. anisopliae* that are confined largely to the haemolymph prior to host death.

Trehalose could be transported intact across the fungal plasma membrane or first be

hydrolysed externally and the glucose monomers transported inside and absorbed. The former strategy would require intracellular trehalase while the latter one would necessitate extracellular fungal enzymes (α -glucosidase and/or acid trehalase). Some fungi e.g. *Mucor* are impermeable to disaccharides (Reyes and Ruiz-Herrera, 1972; Sorrentino et al., 1977), and the corresponding hydrolases must be external to the cell membrane for these substrates to be utilized. The cell-wall fraction in *M. rouxii* constituted 80-90% of the total α -glucosidase activity in hyphal cultures (Sorrentino et al., 1977).

This chapter describes the changes in trehalose-hydrolysing enzyme activity in the haemolymph of *M. sexta* mycosed by *M. anisopliae*, and the partial characterisation of an trehalose-hydrolysing enzyme, α -glucosidase-1, which appears to be produced by the fungus in the haemolymph of infected *M. sexta*. The role of this enzyme in mycosis is discussed.

5.2 Results

Trehalose-hydrolysing enzyme activity in the haemolymph of *M. sexta* infected with *M. anisopliae*

The 5th stage caterpillars were dipped in a suspension of either $1.0 \times 10^6 \text{ ml}^{-1}$ live spores or dead spores. Three days after treatment trehalose-hydrolysing enzyme activity in the haemolymph increased in infected caterpillars in comparison with controls and untreated healthy insects, ca. 100 times higher than the untreated healthy insects (Fig 5.1). The appearance of significant trehalose-hydrolysing enzyme activity in the haemolymph was

associated with a conspicuous increase in glucose concentration during the later stages of infection, x 17 and x 40 the control on day 4 and day 6 respectively (Fig 5.2).

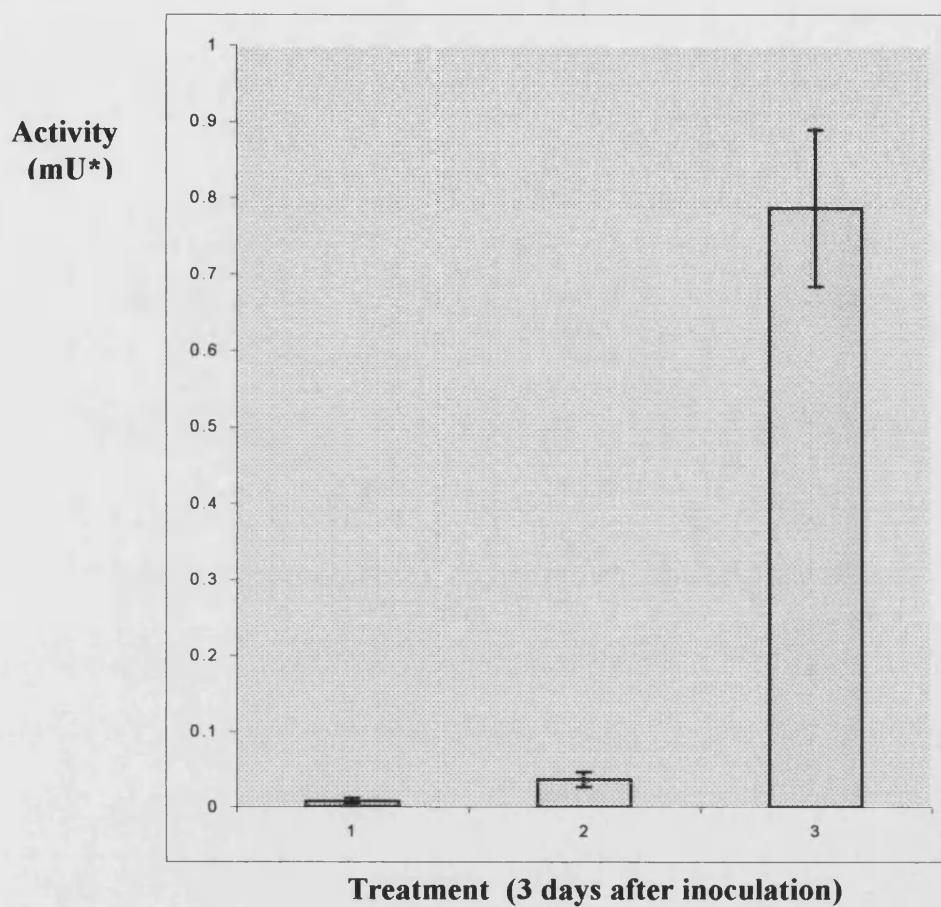


Fig 5.1 Trehalose-hydrolysing enzyme activity of *Manduca sexta* haemolymph (n=10, Mean \pm SE)

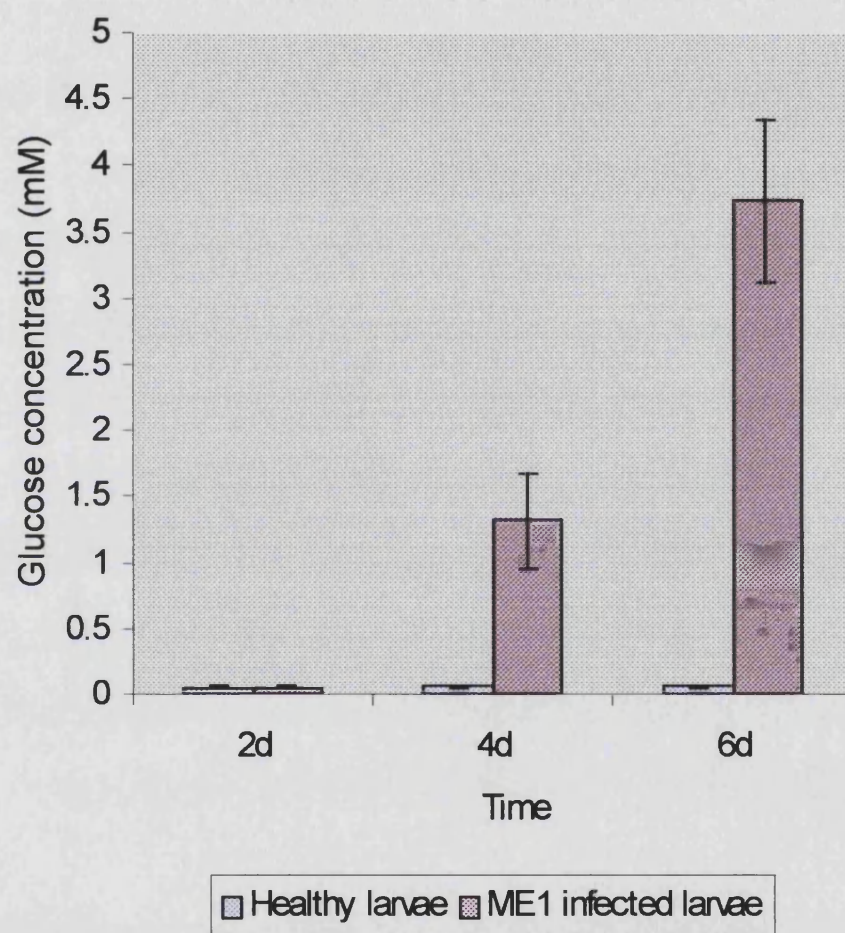
1, Healthy

2, Dipped in 1.0×10^6 ME1 dead spore suspension

3, Dipped in 1.0×10^6 ME1 live spore suspension

U*: 1 unit = 1mM glucose formed. $\text{mg}^{-1}\text{protein.h}^{-1}$

Figure 5.2 Glucose concentration in the haemolymph of *Manduca larvae*
(N=10, Mean \pm SE)



5.2.2 Isozymes of trehalose-hydrolysing enzymes and α -glucosidase isozymes in the haemolymph of *M. sexta* infected with *M. ansopliae*

Isoelectric focusing was carried out in 5% vertical IEF gels that were then stained for trehalose-hydrolysing enzyme activity (vs trehalose) and α -glucosidase (vs maltose) activity using overlay methods. With maltose as substrate, six bands were visualised on IEF gels from the blood of 3d infected caterpillars. Four of these isoforms were acidic (within the range pI 4.5-5.2) and two were neutral. The four acidic isoforms only existed in the haemolymph of infected larvae and were suppressed in 6d infected larvae. The neutral isoforms were also present in haemolymph of dead spore-treated larvae and healthy larvae. One of the acidic isoforms (pI 4.8) was present in 3d and 6d infected larvae and in an *in vitro* culture of the same fungus, but was not found in uninfected larvae (Fig 5.3).

Using trehalose as substrate, there were only four bands on IEF gels of the blood of 3d infected caterpillars. Three isoforms were acidic, within the range pI 4.8-5.5, and one was neutral. Similar to the acidic α -glucosidase isoforms, the acidic isoforms of trehalose hydrolases were only present in the haemolymph of 3d infected larvae, and were suppressed in 6d infected larvae. Once again a pI 4.8 isoform was found in the blood of 3d and 6d infected insects as well as in the fungal culture, while absent from controls (Fig 5.4).

In conclusion, an α -glucosidase isoform present in the haemolymph of infected larvae corresponded to one of the *Metarhizium* α -glucosidase isoforms produced in culture. They both had pIs of ca pH 4.8, and both could degrade trehalose and maltose. In the light of this result, the pI 4.8 isoform was targeted for purification from *in vitro* cultures of the fungus. The degradation of trehalose by fungal secreted trehalose-hydrolysing enzymes resulted in glucose increase in haemolymph of *M. sexta* larvae.

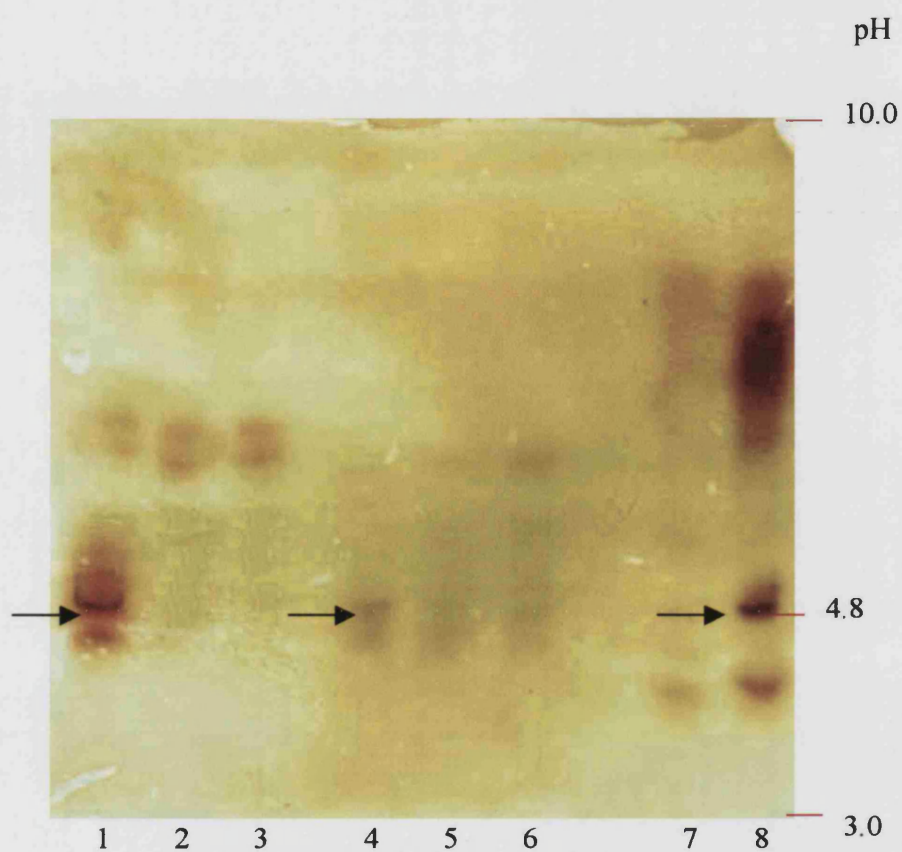


Fig 5.3 α -Glucosidase (vs maltose) isozymes from haemolymph of *M. sexta* and culture of *M. anisopliae* (IEF gel, pH 3 –10)

- 1, Haemolymph of *M. sexta* dipped in live spores (3d)
- 2, Haemolymph of *M. sexta* dipped in dead spores (3d)
- 3, Haemolymph of healthy *M. sexta* (3d)
- 4, Haemolymph of *M. sexta* dipped in live spores (6d)
- 5, Haemolymph of *M. sexta* dipped in dead spores (6d)
- 6, Haemolymph of healthy *M. sexta* (6d)
- 7, 8, ME1 filtrate

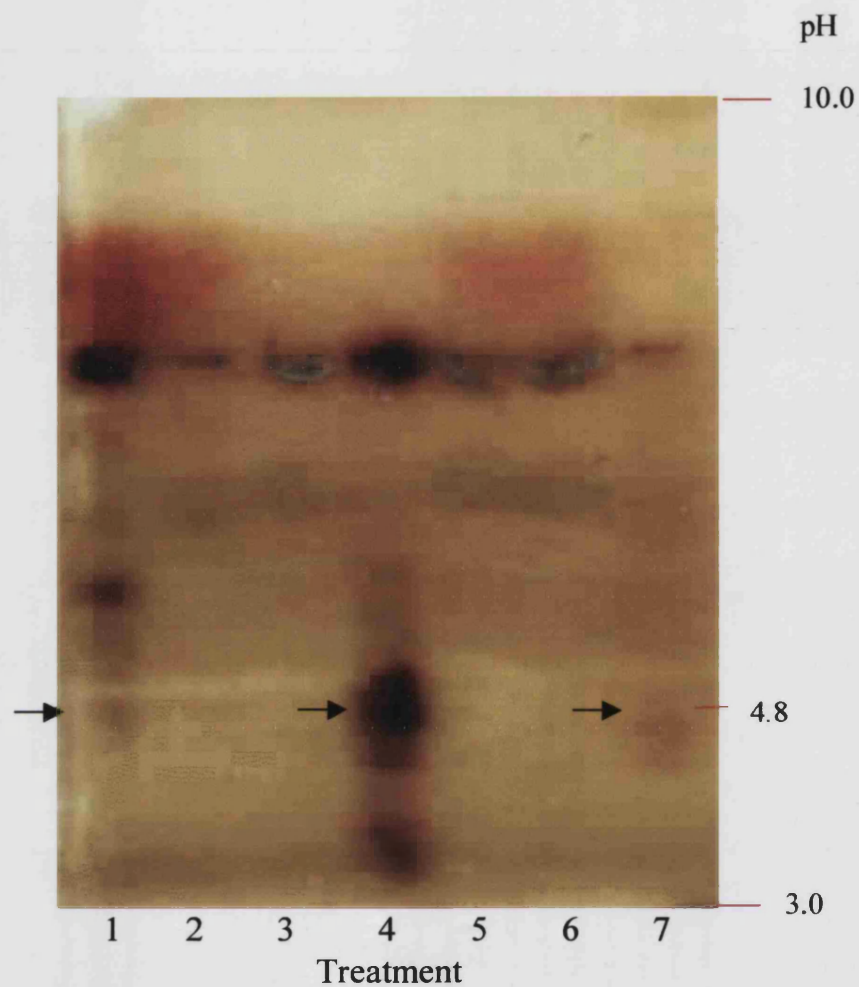


Fig 5.4 Isozymes of trehalose-hydrolysing enzyme (vs trehalose)
from haemolymph of *M. sexta* and culture of *M. anisopliae*
(IEF gel, pH 3.0 – 10)

- 1, Haemolymph of *M. sexta* dipped in live spores (3d)
- 2, Haemolymph of *M. sexta* dipped in dead spores (3d)
- 3, Haemolymph of healthy *M. sexta* (3d)
- 4, ME1 filtrate
- 5, Haemolymph of healthy *M. sexta* (6d)
- 6, Haemolymph of *M. sexta* dipped in dead spores (6d)
- 7, Haemolymph of *M. sexta* dipped in live spores (6d)

5.2.3 Purification and characterisation of α -glucosidase from a culture of *M. anisopliae*

One α -glucosidase isoform (pI4.8) was purified from *Metarhizium anisopliae* culture to homogeneity as determined by silver staining on SDS - PAGE. Purification was achieved through ion exchange (Q-Sepharose), gel filtration (Sephadex G100), narrow range IEF (pH 4.5 - 5.1) and 10-20% gradient PAGE. There was only one α -glucosidase band on the gradient PAGE. This produced a single band, MW ca. 90.5 kDa, on a silver stained SDS-PAGE (Fig 5.5). The progress of each purification step is summarised in Table 5.1.

The optimum pH of the enzyme (designated α -glucosidase-1) was about 6.4, which is similar to the pH of *M. sexta* blood. The enzyme only hydrolysed trehalose and maltose out of eight disaccharides tested (Table 5.2).

To test the ability of α -glucosidase-1 to hydrolyse carbohydrate in haemolymph, 10 ng pure α -glucosidase-1 was incubated with 10 μ l haemolymph of 5th *Manduca sexta* larvae at 27°C for 20min. After incubation, glucose concentration in haemolymph containing α -glucosidase-1 increased more than 1mM. The specific activity of α -glucosidase-1 against substrates in haemolymph was 5.48 mM glucose/ μ g protein/min (Table 5.3), suggesting that α -glucosidase-1 can efficiently degrade disaccharides in insect blood. There was considerable variation in enzyme activity presumably reflecting variation in haemolymph carbohydrate titre between insects.

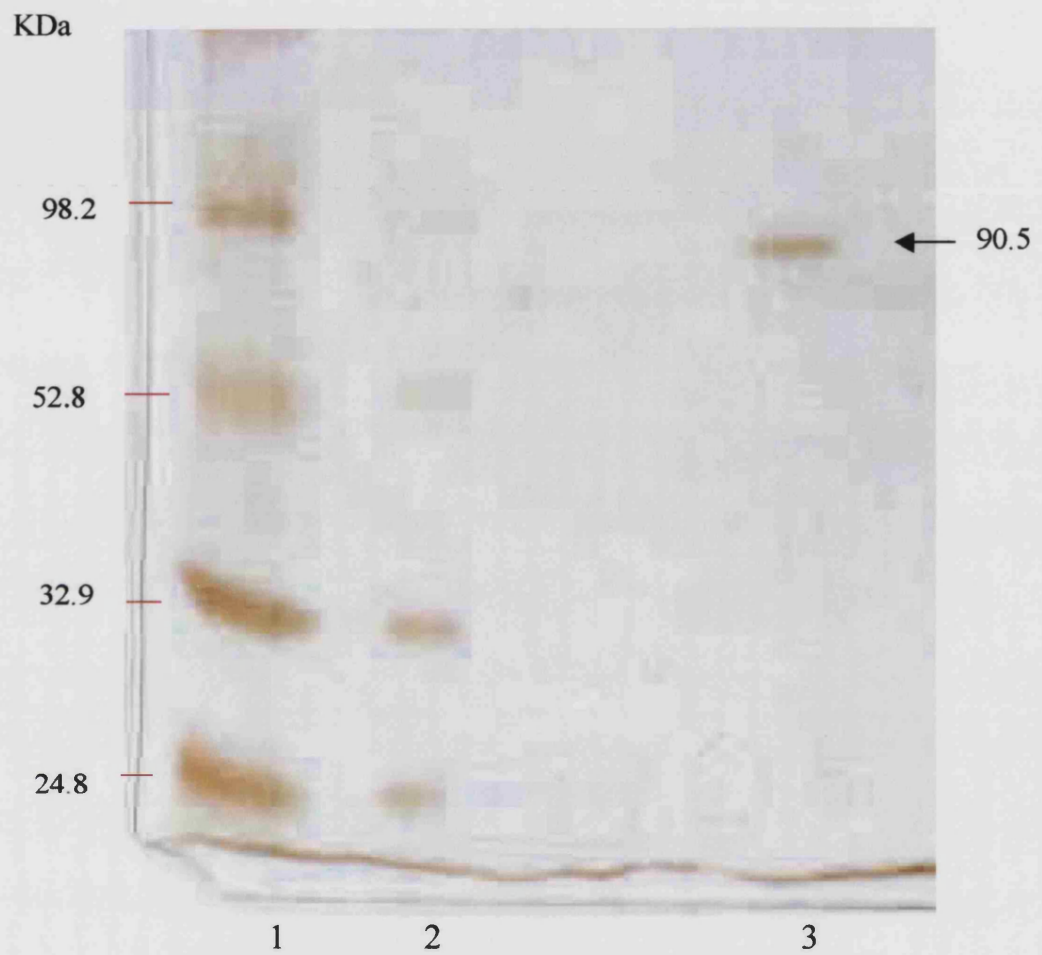


Fig 5.5 α -glucosidase -1 on SDS - PAGE (12%) stained by silver

1, 2, Molecular markers

3, α -glucosidase -1

Table 5.1 Purification of *M. anisopliae* α -glucosidase -1

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Purification Factor	Yield (%)
Mixture of secreted and cell wall bound proteins	964.6	15.38	0.016	—	—
Ion exchange (Mono Q)	26.7	7.93	0.297	18.6	51.6
Gel filtration (G100)	3.93	5.86	1.491	93.2	38.1
IEF gel (pH 4.6-5.1)	0.58	2.97	5.121	320.1	19.3
Gradient PAGE (10-20%)	0.31	2.13	6.871	429.4	13.8

Table 5.2 Substrate specificity profile of α -glucosidase -1
(n=3; Mean \pm SE)

Substrate	Cellobiose	Lactose	Maltose	Sucrose	Trehalose	pN- α -gal	pN- α -glu	pN- β -glu
^a Specific Activity (U)	0.000	0.000	3.462 \pm 0.152	0.000	6.843 \pm 0.664	0.000	0.000	0.000
^b Relative activity (%)	0.000	0.000	50.59	0.000	100.00	0.000	0.000	0.000

^aSpecific activity: 1 Unit = 1 mM glucose generated $\text{mg}^{-1}\text{protein}\cdot\text{min}^{-1}$, and 10ng α -glucosidase was used in each reaction.

^bRelative activity is expressed as percentage (%) relative to the enzyme activity displayed with 10 mM trehalose.

pN- α -gal : p - Nitrophenyl- α -galactopyranoside; pN- α -glu : p - Nitrophenyl- α -glucopyranoside;

pN- β -glu : p - Nitrophenyl- β -glucopyranoside.

Table 5.3 α -Glucosidase-1 activity against substrates in haemolymph

(n=3, mean \pm SE)

Control	Glucose increase	α -glucosidase-1 activity
(glucose level, mM) ^(a)	(glucose level, mM) ^(b)	(mM glucose/ μ g protein/min) ^(c)
5.74 \pm 1.88	1.10 \pm 0.41	5.48 \pm 2.03

(a): glucose level in the mixture of *M. sexta* haemolymph(10 μ l) and dH₂O (1 μ l).

(b): increased glucose level in the mixture of *M. sexta* haemolymph(10 μ l) and 10 ng α - glucosidase-1(1 μ l) after 20 mins incubation at 27 °C.

(c): α - glucosidase activity against substrates in the haemolymph of *M. sexta*.

5.3 Discussion

A large amount of trehalose-hydrolysing enzyme activity appeared in the haemolymph of caterpillars of *M. sexta* infected with the fungus *M. anisopliae*. At least a proportion of the activity appears to derive from the fungus. Isoforms of an enzyme with trehalose-hydrolysing activity were found in inoculated *M. sexta* larvae during infection which were absent from control insects. One of the extra trehalose-hydrolysing isoforms also hydrolysed maltose and therefore may be designated as a α -glucosidase rather than a trehalase which is specific for trehalose (Kelly and Fogarty, 1983). This enzyme had a pI similar to one of the fungal isoforms in culture as well as the ability to degrade both trehalose and maltose. Thus this isoform (pI 4.8) found *in vivo* appears to come from the fungus. The origin of other extra isoforms is not clear.

α -Glucosidase usually has a broad substrate specificity and is capable of hydrolysing a wide range of α -D-glucosides with maltose (α -1,4) and sucrose (α -1,2) being the preferred substrates (Kinsella *et al.*, 1991). Many α -glucosidases hydrolyse α -1,4, α -1,6-, α -1,1, α -1,2 and α -1,3-linked disaccharides or oligosaccharides with varied efficiency (Yamamoto, 1988; Kinsella *et al.*, 1991; Tibbot *et al.*, 1998). The purified α -glucosidase-1 only hydrolysed trehalose (α -1,1) and maltose (α -1,4) out of eight disaccharides tested. This indicates that α -glucosidase-1 has a narrow substrate specificity in comparison with enzymes from other sources. Both the substrates of this enzyme, trehalose and maltose, are found in haemolymph, and trehalose is the principal haemolymph sugar in many

species of insects (Wyatt, 1967). This property of α -glucosidase-1 maybe especially important for the degradation of the principal haemolymph sugars. α -Glucosidase-1 had an optimal pH similar to that of haemolymph of *M. sexta*. Furthermore pure α -glucosidase-1 released large amounts of glucose following incubation with *Manduca* haemolymph.

Glucose concentration, normally low in *M. sexta* haemolymph, increased dramatically during mycosis, with a coincident decline in trehalose (Phalaraksh, 2000); it seems that hydrolysis of trehalose by extracellular fungal trehalase and α -glucosidase outstrips the ability of the fungus to absorb the resulting glucose. Interestingly, despite the large increase in glucose concentration there was no corresponding increase in osmotic pressure (Xia *et al.*, unpublished observation). Therefore the mycosed insect is still able to keep one aspect of its haemolymph physiology in balance during infection.

Thus extracellular carbohydrases may contribute to pathogenesis in several ways. First, by hydrolysing trehalose they provide glucose for fungal nutrition. Secondly the excess glucose may be detrimental to the insect. Elevated haemolymph glucose may be in part responsible for the decrease in feeding in mycosed insects that has been recorded by many authors (e.g. Seyoum *et al.*, 1994; Bell, 1974). Consistent with these claims, injection of pure α -glucosidase-1 into caterpillars caused an increase in haemolymph glucose and a significant reduction in feeding (Xia, Clarkson and Charnley, unpubl). Adaptation by the fungus to reduce host feeding may help to weaken the insect, reduce defence responses and hasten death.

CHAPTER SIX

MOLECULAR CLONING, CHARACTERISATION AND EXPRESSION OF A NEUTRAL TREHALASE FROM THE INSECT PATHOGENIC FUNGUS *METARHIZIUM ANISOPLIAE*

6.1 Introduction

Trehalases [EC 3.2.1.28] hydrolyse the non-reducing disaccharide of glucose, trehalose [α -D-glucopyranosyl (1,1)- α -D-glucopyranoside]. Fungal trehalases are categorised into acid and neutral trehalases according to their pH optimum (Thevelein, 1984). Acid trehalases exhibit their maximal activity around pH4.5, whereas neutral trehalases exhibit their maximal activity around pH 7.0. They appear to play very distinct roles in the fungal cell.

Acid trehalase proteins have been localised to the cell wall in filamentous fungi (Hecker and Sussman, 1973; d'Enfert and Fontaine, 1997), and these enzymes are involved in the assimilation of extracellular trehalose as a carbon source (d'Enfert and Fontaine, 1997). Neutral trehalases are regulatory and are localised in the cytosol (App and Holzer, 1989). Filamentous fungi accumulate large quantities of trehalose during spore formation or in

response to heat shock, and this trehalose pool is rapidly degraded during growth resumption (Thevelein, 1984; d'Enfert, 1997). Neutral trehalases in filamentous fungi catalyse the mobilization of intracellular trehalose during conidial germination (d'Enfert et al., 1999). Fungal neutral trehalase controls trehalose hydrolysis in response to multiple stress conditions, including nutrient limitation. Neutral trehalase null-mutants in *Emericella nidulans* failed to mobilise trehalose during conidial germination. Analysis of these mutant strains suggests that trehalose mobilization can contribute some of the energy requirements of spore germination under limiting external carbon, and that trehalose can act as a thermoprotectant in conidia of filamentous fungi (d'Enfert et al., 1999).

Metarhizium anisopliae (*Ma*) is a filamentous entomopathogenic Deuteromycete fungus which is used commercially as a biological control agent (Charnley, 1997). In common with other entomopathogenic fungi, trehalose plays an important part in the stress response of *Metarhizium* (Hallsworth and Magan, 1996). Furthermore, trehalose, the main disaccharide in the haemolymph of host insects, is a readily available energy resource during mycosis. Neutral trehalase may play a role in utilisation of haemolymph trehalose following direct uptake by disaccharide permeases.

In order to facilitate a study of the role of a neutral trehalase in mycosis by *M. anisopliae*, we have cloned and sequenced a neutral trehalase gene of *Ma* and expressed it in *Saccharomyces cerevisiae*. We also report here the expression of a neutral trehalase gene during infection of *Manduca sexta* by *M. anisopliae* strain ME1.

6.2 Results and discussion

6.2.1 Cloning and sequencing of the *ntl1* gene

Two degenerate sense primers (ntreF1/ntreF2) and one degenerate antisense primer (ntreR) were designed to correspond, respectively, to two regions conserved in neutral trehalases from yeasts and filamentous fungi. However, only the combination of sense primer ntreF1 and an antisense primer ntreR generated a product of the predicted size. Using these two primers, a 1.0kb fragment was amplified by PCR from genomic DNA of ME1. The 1.0kb PCR product was cloned and sequence comparison confirmed that it was a fragment of a neutral trehalase gene (Fig 6.1). The deduced amino-acid sequence revealed significant identity to corresponding regions of neutral trehalases from *Saccharomyces cerevisiae* (60% identical amino acids) and *Magnaporthe grisea* (81% identical amino acids) and *Emericella nidulans* (78% identical amino acids). The 1.0kb PCR fragment was used to screen an λ EMBL3 genomic library of *M. anisopliae* DNA. A 5.8kb XhoI fragment hybridising to the 1.0kb fragment was subcloned into pBluescript. The nt sequence of a neutral trehalase gene in the subclone was determined by overlapping sequencing starting from the two ends of the 1.0kb sequence.

The nt sequence of a 3.4-kb region of a 5.8kb XhoI subclone was determined (Fig. 6.2) and found to contain an ORF of 2211bp in addition to 1028bp upstream and 18bp downstream UTR. The 5'UTR contains two STREs (stress responsive elements,

CCCCT). STREs have been shown to mediate transcriptional activation in response to various stresses in *S. cerevisiae*, especially heat, osmotic stress, low pH and nutrient starvation (Siderius and Mager, 1997). Potentially the STRE elements perform a similar function in *M. anisopliae*. Analysis of this sequence revealed the ORF was interrupted by three putative introns of 52, 61 and 59bp. The location of these introns was corroborated by comparison with the nt sequence of the neutral trehalase cDNA. The junction between intron follows the “GT-AG rule” (Breathnach and Chambon, 1981). Southern hybridisations (Fig. 6.3) detected the presence of a single band in each digest, suggesting that the neutral trehalase gene exist as a single copy in the genome.

Figure 6.1 Comparison of the deduced amino acid sequence of the 1kb *ntl1* gene fragment with fungal neutral trehalase sequences

The aligned amino acid sequences are: 1kb *ntl1*, the 1kb *ntl1* gene fragment of ME1; TREB_MAGGR, *Magnaporthe grisea* (Sweigard *et al.*, 1997); TREB_EMENI, *Emericella nidulans* (*Aspergillus nidulans*, d'Enfert *et al.*, 1999); TREB_YEAST, *Saccharomyces cerevisiae* (Kopp *et al.*, 1993).

- * - single, fully conserved residue
- * - conservation of strong groups
- * - conservation of weak groups
- * - no consensus

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1kb ntl1      VPGGRFNELYGWDSYMESLGLLVNDRVDLAKSMVLNFCFCIEHYGKILNATRSYYLGRSQ
TREB_MAGGR   VPGGRFNELYGWDSYMESLGLLVNDKVYLAKSMVLNFCFCIKHYGKILNATRSYYLGRSQ
TREB_EMENI   VPGGRFNELYGWDSYMESLGLLVNDRVDLAKSMVLNFCFCIKHYGKILNATRSYYLGRSQ
TREB_YEAST   VPGGRFNELYGWDSYLMALGLIESNKVDVARGMVEHFIFEIDHYSKILNANRSYYLGRSQ

1kb ntl1      PPFLTDMALRVYEKIK--HEPDAKEFLRRSILAAIKEYHSVWTCPEPRLDPTGLSRYRPE
TREB_MAGGR   PPFLTDMALRVYDKIR--HEPDATEFLRTAILAAIKEYHSVWVAEPRLDPTGLSRYRPE
TREB_EMENI   PPFLTDMALRVYDRIQ--NEPGAMDFLRHAILAAIKEYYSVWMAEPRLDPTGLSRYRSP
TREB_YEAST   PPFLTDMALLVFEKIGGKNNPNAIQLLKRAFRAAIKEYKEVWMSSPRLDSLTGLSCYHSD

1kb ntl1      GLGVPPETEAGHFVHILEPYVKKHNMFEKFEVRAINYGEIKEPELDDYFMHRAVRESGH
TREB_MAGGR   GAGVPPETEADHFLHILEPYVKKHNMTEKFEVEAYNFGRIPEPELDKYFLHRAVRESGH
TREB_EMENI   GIGVPPETEASHFLHLLTPYAEKHGMFEKFEVQAYNYGKVKEPELDEYFMHRAVRESGH
TREB_YEAST   GIGIPPETEADHFDITLLPYAEKYNVTLEKRLRYLYNEGMIKEPKLDAFFLHRAVRESGH

1kb ntl1      DTTYRFEGICADLATIDLNSLLFKYETDIARTIRNVFNDKLVIPGEFCDRTPTYQPGEVLA
TREB_MAGGR   DTSYRLEGVCADLATVDLNTLLFKYETDIARTIRNVFGDKLVIPAEYCVGS-LQPGQVET
TREB_EMENI   DTSYRLERVCGNLATVDLNSLLYKYEVDIARVIRVYFKDKLEIPVEFRTPA-TKDIQSES
TREB_YEAST   DTTYRFEGVCAYLATIDLNSLLYKYEKDIAFVIKEYFGN-----EYKDEN---DGTVTD

1kb ntl1      SAAWDRRAKRRKLTVDKLMWDEEQGVFFDYDTAKRERCNYESSTTFWALWAGIATPKQAA
TREB_MAGGR   SAIWDRRSKRRKLAIKYLWNEEAGMYFDYDTAKRQCCNYESCTTFWALWAGVASPKQAA
TREB_EMENI   SSVWDRRAKRRKMRMDTYLWDEEKGMFYFDYDTVKQERTNYESATTLWAMWAGLVTPRQAS
TREB_YEAST   SEHWEEELAE LRKTRINKYMWDEDSGFFYYNTKLKCRTSYESATTFWSLWAGLATEEQAK

1kb ntl1      DMVKKGLPRFEAYGGLLAGTEKSRGDIGLERPNRQWDYPYGW
TREB_MAGGR   IMVTRALPKFEAYGGLLSGTEESRGQIGLDRPNRQWDYPYGW
TREB_EMENI   AMITKALPRFEFEGGIVSGTEESRGAVGLNRPTQWDYPYGW
TREB_YEAST   ITVEKALPQLEMLGGLVACTEKSRGPISIDRPIRQWDYPYFW

```

6.2.2 The NTL1 protein

The *ntl1* gene encodes a protein of 737 amino acids with a calculated molecular mass of 83.1kDa. The deduced amino acid sequence of the protein displays high similarity to the sequences of the enzyme from filamentous fungi, *M. grisea* (78%) and *E. nidulans* (64%). There is extensive identity (52%) between NTL1 and the neutral trehalase from *S. cerevisiae*. There is considerably less identity (13%) to the sequence from an insect (silkworm, Su *et al.*, 1993). A cAMP dependent-phosphorylation consensus site (Kemp and Pearson, 1991) and a putative calcium binding site (Geiser *et al.*, 1991) exist in the amino-terminal domain (Fig. 6.2), this suggests that the neutral trehalase gene encodes a regulatory trehalase. However, the functionality of these sites remains to be demonstrated. No signal peptide was found in the sequence, indicating *ntl1* encodes an intracellular protein, this is common with neutral trehalases from other organisms. Although two potential N-glycosylation sites N-X-S/T were found, but it is unlikely that the enzyme is glycosylated because it is an intracellular enzyme.

Figure 6.2 The 3408 nt sequence of neutral trehalase gene of *Ma* ME1 and deduced aa sequence.

Numbers on the left refer to the position in the adjacent nt or aa sequence.

Lower-case letters represent non-coding sequences.

The asterisk indicates the TGA stop codon.

Underlined bold aa regions is the cAMP dependent-phosphorylation consensus site (Kemp and Pearson, 1990).

Underlined aa regions denote the putative Ca^{2+} -binding site (Geiser et al., 1991).

Dots show N- glycosylation sites (N-X-S/T) at positions 325, 686.

STRE element (5'-CCCCT-3', Siderius and Mager, 1997) is underlined bold lower case letters.

Oligos used for southern analysis of RT-PCR are indicated by dashed arrows above the nt sequence.

```

-1020      -1048 aatatcttgtgggaaatgcatggaatac
-960      gcttgatatataatgtggcatttcacaatttatttagtcagaattcacaaagataatca
-900      gaaccattcacacaatgcttttcacgtattttgaatgttcaaggatgtacgtctgaatga
-840      tgatatcgtgaaaccacgacgcagagtaccgatgcagtcaggagtgacctgcagaacttcg
-780      gaagtgggagccgcccgatgtcggcagctgtacagggcaacacattaccgaagaaagagtg
-720      atgatgcgagtcctcatcagaatttgatagcctagtattcggtagcgagtgccctgaagtggcattgatca
-660      cgttggatgaatgcattcatcgcaagttgtagccgagtgccctgaagtggcattgatca
-600      aattcgacgcccagatgccgaatgcggtgcggagccgagacccttcaatgtttggcctcc
-540      catctccccatcctgggtcccggtcaacgggtcggccactttaccagatcaacttgacat
-480      ttgaatgggtggggcctagccagcctctgcaggggtatcgtcaccgccccttctctgggact
-420      ggaaccgcaagtctttccgataggaacaaagccgtctgcgtgctttttgcctctccaaa
-360      ctatccatctgacatctcttgtcatcaattgaggtctctccgggaacatgcaagagcccc
-300      cacgacgtgggattgagtccttcgtagtacgaatctgacactagacatcagtcagctacc
-240      cgcaagcccccctccaacgtccccactcccagccagtccccccttctgtgtcggcgacacett
-180      tttgccttttttttttttttgacgacagagcttaactcttagctcatctcgccatcgctcc
-120      ccgtgacgcaaacgcggttccttcagcctgccgaatagttcaactttaggttttattgc
-60      atttttgattatttattcatcccccatctgctactgtcgctacaagcctcctttcatttc
      tgcacggcagtcgtcttgtggccaccttctcgctccgtcagcctgcgccatcgctcatagtc

      - - - - -
1      ATGGCGGGAACGACAAACCACCGAGCGCGAGGCTCGGAGGACTATAATGTGTTTGACGAC
1      M A G T T N H R A R G S E D Y N V F D D
      →
61      GCAAAGACATACTATGCTTCTGAAGAGCGACATCAAAACCGATTGGGTCCCGTACACGT
21      A K T Y Y A S E E R H Q N R F G S R T R

```


121 ACATACTCCCAG**gt**atttgctcgacttgctctgcgacgccgtggatacaaatttgactgtgc
 41 T Y S Q

181 at**ag**AACAGCTTGATGAAGCAGTTTGAACGAAACGGCCTGCGTGAACCATACAGAAGGGG
 45 N S L M K Q F E R N G L R E P Y R R G

241 CAGCCACG**gt**aagacttcttggtttttcttctctgctcgagcgctatggatatatctaattc
 64 S H D

301 tggaaat**ag**ACGAGAGTTCGCTGCCCCAGGGCCGCCGATTCTCATCCAGGTCGACCCTA
 67 E S S L P Q G R R F L I Q V D P T

361 CTCTCGAAAGCTTGCAAGCCCAAGAAGACACCGATGGCAATATGCAAATTACCATCGAGG
 84 L E S L Q A Q E D T D G N M Q I T I E D

421 ACGAAGGCCCCAAAGTACTCTCTCTACGAACCGCGGCTTCTGCCGGCCACAATCGTTTCG
 104 E G P K V L S L R T A A S A G H N R F E
 481 AAGTCCGAGGCACATATATGCTCTCCAATCTGCTGCAGGAATTGACCTTGGCGAAAGAAT
 124 V R G T Y M L S N L L Q E L T L A K E Y

541 ACGGGCGGAAACAAATCGTCCTCGACGAGGGTCGGCTCAATGAGAATCCAGTCGATCGTC
 144 G R K Q I V L D E G R L N E N P V D R L

601 TGTCCCGCTGATCCGTGACCACTTCTGGGATGGCCTGACTAGACGTATCGATGCATCCA
 164 S R L I R D H F W D G L T R R I D A S S

661 GTATCGAAATAGCCGCGGAGACCCCAAGGATTGGACGGATGATCCTCGACCACGGATCT
 184 I E I A A R D P K D W T D D P R P R I Y

721 ACGTCCCTGCCGGAGTACCGGAGCAATATGAATACTACAAGCAAGTTGCCGAGGAGCGGC
 204 V P A G V P E Q Y E Y Y K Q V A E E R P

781 CGGAGATTCTGTCTAGATGTCCAGCAGCTCCCCGAAAAGATCACGCCCGAGTTGATCCGGG
 224 E I R L D V Q Q L P E K I T P E L I R D

841 ACATGAACGAGAAGCCCGGATTGCTGGCGGTGCGAAACCGAACAAGTCGTGGACCCTGAAA
 244 M N E K P G L L A V E T E Q V V D P E T
 901 CGGGTCGAAAAACACTACAGGGCCTCCCCTTTGTAGTGCCTGGAGGGAGATTCAACGAAT
 264 G R K T L Q G L P F V V P G G R F N E L
 961 TATACGGCTGGGACAGCTACATGGAGTCACTTGGCCTGTTGGTCAACGACAGGGTTGACT
 284 Y G W D S Y M E S L G L L V N D R V D L
 1021 TGGCAAAGTCAATGGTGCTGAATTTTTGTTTCTGCATTGAGCACTACGGAAAGATACTCA
 304 A K S M V L N F C F C I E H Y G K I L N
 1081 ATGCTACCCGCTCGTACTATCTCGGCCGATCGCAGCCCCCATTCCTAACCGACATGGCAT
 324 A T R S Y Y L G R S Q P P F L T D M A L
 1141 TGCGCGTGTACGAAAAGATCAAGCATGAACCGGATGCCAAGGAATTCCTTAGACGGTCGA
 344 R V Y E K I K H E P D A K E F L R R S I
 1201 TACTAGCTGCCATAAAGGAGTACCACAGTGTCTGGACTTGTGAGCCCCGGCTGGACCCGG
 364 L A A I K E Y H S V W T C E P R L D P V
 1261 TCACTGGTTTGTGCGAGGTATCGGCCAGAGGGCCTGGGTGTGCCTCCGGAAACGGAGGCTG
 384 T G L S R Y R P E G L G V P P E T E A G
 1321 GCCATTTTGTGCACATTCTGGAGCCGTATGTCAAAAAGCACAAATATGGAGTTTAAGGAGT
 404 H F V H I L E P Y V K K H N M E F K E F
 1381 TTGTTGCGGCATACAACCTATGGCGAGATCAAGGAGCCCGAATTGGATGACTATTTTATGC
 424 V R A Y N Y G E I K E P E L D D Y F M H
 1441 ACGATCGAGCAGTGCAGAGTCTGGGCATGACACTACGTATCGATTGGAAGGCATTTGTG
 444 D R A V R E S G H D T T Y R F E G I C A
 1501 CCGACCTTGCCACTATTGATCTCAACTCACTCTTGTTCAAATACGAAACCGACATTGCGC
 464 D L A T I D L N S L L F K Y E T D I A R

1561 GCACAATCCGCAACGTTTTTTAACGACAAGCTTGTGATCCCGGGCGAGTTCTGCGATAGGA
 484 T I R N V F N D K L V I P G E F C D R T
 1621 CGCCGTATCAGCCGGGGGAGGTGCTGGCGTCTGCAGCTTGGGATCGACGCGCGAAGCGAC
 504 P Y Q P G E V L A S A A W D R R A K R R
 1681 GCAAGCTCACTGTTGACAAGTTGATGTGGGACGAAGAACAGGGAGTCTTTTTTGATTACG
 524 K L T V D K L M W D E E Q G V F F D Y D
 1741 ACACGGCAAAGCGGGAGCGATGCAACTATGAAAGCTCAACCACGTTTTGGGCTCTGTGGG
 544 T A K R E R C N Y E S S T T F W A L W A
 1801 CGGGCATTGCGACGCCGAAGCAGGCGGCCGACATGGTTAAGAAGGGGTTGCCGCGATTTG
 564 G I A T P K Q A A D M V K K G L P R F E
 1861 AAGCGTACGGTGGTCTGTTGGCTGGCACTGAGAAGTCTCGCGGGGACATCGGGCTTGAGC
 584 A Y G G L L A G T E K S R G D I G L E R
 1921 GCCCCAACCGACAATGGGACTACCCGTACGGCTGGGCTCCTCAGCAGATGCTTGCCTGGA
 604 P N R Q W D Y P Y G W A P Q Q M L A W T
 1981 CTGGTCTGCTCCGGTATAGCTTCACAGAAGAGGCCGAGCGTCTGGCCTACAAGTGGCTGT
 624 G L L R Y S F T E E A E R L A Y K W L F
 2041 TTATGATTACCAAGGCGTTTGTGATTTCAACGGTGTCTGTTGTTGAAAAGTACGACGTGA
 644 M I T K A F V D F N G V V V E K Y D V T
 2101 CGCGACCAGCTGATCCTCATCGCGTGGATGCCGAGTATGGTAACCAAGGGCTGGACTTTA
 664 R P A D P H R V D A E Y G N Q G L D F K
 2161 AAGGTGTTGCCAAAGAAGGgtaggttttatctcgtcgtgatggcctttgctgctacggtcg
 684 G V A K E G
 2221 acgctgacaagagtctagGTTTGGCTGGGTAAATGCCAGTTATGTGTATGGTCTGCAGAT
 690 F G W V N A S Y V Y G L Q I

2281 TGTAATGCACATATGCGTCGTGCCCTGGGTACCCTGACACCGTATCCGACGTTTATCAA
704 V N A H M R R A L G T L T P Y P T F I K
2341 GGCGATTGAACAGAGTTTGGAGAAGGAATTGGCTGATTGCCGtgagtagcggtatgata
724 A I E Q S L E K E L A D L P * 737

Figure 6.3 Southern analysis of the chromosomal *Ma* neutral trehalase gene

Ma DNA digested with *Xho*I, *Sma*I, *Not*I, *Eco*RV and *Bam*HI restriction enzyme was hybridised with the radiolabeled 1.0kb PCR product amplified from *Ma* DNA with primers ntreF1 and ntreR.

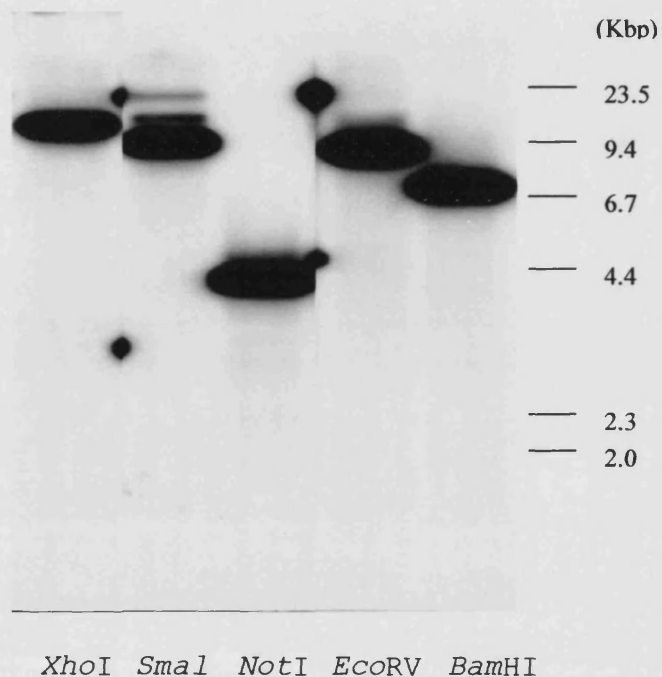


Table 6.1 Expression of *Ma* neutral trehalase by transformed *S. cerevisiae*

(n=3, mean \pm SE)

Plasmid	Medium	Specific activity (U / mg of total protein)
pYtre	YPD	0.259 \pm 0.030 ^a
pYtre	YPGal	1.077 \pm 0.093 ^b
pYES2	YPD	0.269 \pm 0.012 ^a
pYES2	YPGal	0.267 \pm 0.026 ^a

Values with different superscript letters are significantly different ($p \leq 0.05$).

pYtre : construct for *ntl1* expression (a *ntl1* cDNA fragment was inserted into the *Bam*HI and *Xho*I sites of the pYES2 expression vector)

pYES2 : expression vector without insert

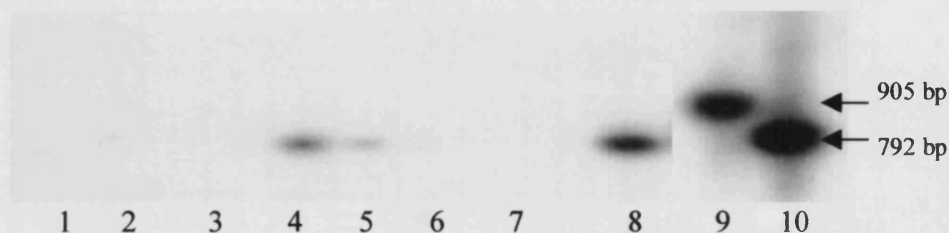
YPD : growth medium for yeast using 1% glucose as carbon source

YPGal: inducing media using 1% galactose as carbon source to induce the production of neutral trehalase from the GAL promotor.

6.2.4 Expression of the *Ma* neutral trehalase gene during mycosis

Trehalose is the main blood sugar of insects and an important energy source for insect tissues (Wyatt, 1967). This disaccharide is synthesised mainly by fat body and released rapidly into the haemolymph with minimum storage within the fat body. To utilise the trehalose for fungal growth, *Ma* may secrete trehalose-degrading enzymes to hydrolyse trehalose to glucose or it can take the intact trehalose molecule into cell and then degrade it by intracellular neutral trehalase. To investigate the possibility of the involvement of neutral trehalase in utilisation of the trehalose within the haemolymph of the insect host during infection, *Ma* strain ME1 was grown in cell-free haemolymph from 5th instar *Manduca sexta* larvae and insects were infected by dipping in ME1 spore suspension. *Ma ntl1* gene expression was analysed by a Southern blot of RT-PCR (Fig. 6.4). Southern analysis indicated that the *Ma ntl1* gene was expressed *in vitro* in liquid culture of insect haemolymph and *in vivo* in haemolymph of *M. sexta* in the early stages of infection. Probably due to the decline in trehalose concentration in haemolymph of mycosed larvae (Phalaraksh et al., 2000), expression of *ntl1* was low in the later stages of infection.

Figure 6.4 Southern analysis of RT-PCR products corresponding to the *Ma ntl1* transcript



Lanes 1-3 correspond to mRNA obtained from 2, 4, 6 day 5th instar *M. sexta* larvae respectively.

Lanes 4-6 correspond to mRNA obtained from ME1 infected 5th instar *M. sexta* larvae 2, 4, 6 days after inoculation respectively.

Lane 7 corresponds to a PCR product obtained using primers RTF and RTR, but using *M. sexta* genomic DNA as a template instead of a reverse transcription product.

Lane 8 corresponds to RNA of ME1 grown in cell-free haemolymph of 5th instar *M. sexta* larvae at 26 °C for 2 days.

Lane 9 corresponds to a PCR product obtained using primers RTF and RTR, but using *Ma* genomic DNA as a template instead of a reverse transcription product.

Lane 10 corresponds to a PCR product obtained using primers RTF and RTR, but using cloned cDNA as a template instead of a reverse transcription product. This product was used as a probe.

6.2.5 Conclusions

- (1) Degenerate oligos based on consensus neutral trehalase sequences were used to clone a neutral trehalase gene from *Metarhizum anisopliae* isolate ME1.
- (2) The gene, containing three introns of 52, 61 and 52bp, encodes a protein of 737aa with a calculated molecular mass of 83.1kDa.
- (3) The aa sequence of NTL1 shows 78% identity to the neutral trehalase sequence from the filamentous, phytopathogenic fungus *Magnaporthe grisea* (rice blast fungus).
- (4) Southern analysis indicates that the gene exists as a single copy in the genome.
- (5) The *Ma ntl1* gene was actively expressed as an intracellular form in yeast.
- (6) Southern analysis of RT-PCR indicated *Ma* neutral trehalase gene was expressed *in vivo* in the early stage of infection of *M. sexta* larvae and *in vitro* in liquid culture of *M. sexta* haemolymph.

CHAPTER SEVEN

GENERAL DISCUSSION

Entomopathogenic fungi have evolved to penetrate the cuticle of insects and overcome host immune defences both in the integument and in the haemolymph. Growth of the fungus prior to host death is often confined to the haemolymph. The haemolymph of insects is a complex aqueous medium containing a wide range of inorganic ions, proteins and organic acids. There are particularly high concentrations of sugars, amino acids, and organic phosphates (Phalaraksh, 1999). In order to develop in the haemolymph, the fungus must either be able to take readily available nutrients up directly or mobilise host metabolites by secretion of hydrolases. Subsequent changes in the composition of the haemolymph may be both favourable to the fungus and detrimental to the host.

Carbon, nitrogen, sulphur and phosphorus, as the major mineral nutrient elements, must be essential for growth and development of entomopathogenic fungi. The acquisition of phosphorus and carbon has been the subject of this study.

Phosphorus is one of the major mineral nutrient elements of *Metarhizium* and is essential for growth

Phosphorus is an essential element for all cells, being present in nucleic acids and many other organic molecules, and is important in energy metabolism (Jennings, 1995). The phosphorus content of fungal mycelium remains relatively constant at between 0.26 and 0.31%, if the culture is harvested before all carbon sources are consumed. Orthophosphate plays a central role in the physiology and biochemistry of phosphorus metabolism in fungi (Jennings, 1989). Extensive depletion of most phosphorus compounds of the cell is incompatible with continued growth (Beever and

Burns, 1980). In this study, the growth of *M. anisopliae* in cell-free haemolymph *in vitro* was enhanced in a dose dependent manner by addition of exogenous phosphate, indicating that phosphate is a limiting factor for growth in the haemolymph of larvae.

***Metarhizium* secretes acid phosphatases to utilise organic phosphates**

There are at least two phosphate transporters in fungi which provide flexibility for phosphate uptake (Beevers and Burns, 1980). There is, however, little inorganic phosphate in insect haemolymph. Most phosphorus is tied up in organic phosphates e.g. glucose-1-phosphate and glucose-6-phosphate (Wyatt, 1961, Pannabecker et al., 1992). The concentration of organic phosphates in the haemolymph of *Bombyx mori* was 32-64mM, 16-32 times the concentration of inorganic phosphate. A similar situation exists in 5th instar *Manduca* haemolymph. Though P-monoesters can be taken up intact in some strains of *S. cerevisiae*, in most fungi the specific transport systems required are not present. Fungi are able to utilise exogenous P-monoesters as sources of phosphate, but utilisation of these esters undoubtedly involves their enzymatic hydrolysis with the release of phosphate (Beevers and Burns, 1980). Intact cells of *S. cerevisiae* hydrolyse glucose-1-phosphate and glucose-6-phosphate with the release of glucose and phosphate into the medium (Rothstein and Meier, 1949), indicating the presence of externally located phosphatases. This appears to be the general rule among fungi (Jennings, 1995). Studies with hypo and hyperproductive mutants established the importance extracellular acid phosphatase in the growth of yeast on P-monoesters at pH 6.0 (Hanschke *et al*, 1978). It is clear, however, that fungi with both acid and alkaline extracellular phosphatases have a greater ability to use P-esters over a wide range of environmental pHs than species with only one.

The utilisation of organic phosphate in the haemolymph of host insects by *Metarhizium*

Organic phosphates, principally acid soluble sugar phosphates such as glucose-6-phosphate and α -glycerophosphate, with small amounts of phospholipid and phosphoprotein, predominate over inorganic phosphate in insect haemolymph (Wyatt, 1961). *Metarhizium* is confined to the haemolymph of *Manduca* and *Schistocerca* before death. In the early stage of infection, the orthophosphate in the haemolymph appears to be sufficient for fungal growth. As mycosis progresses, the increase in fungal biomass presumably requires an additional source of phosphate. The late appearance of fungal extracellular acid phosphatase during the infection of *M. sexta* by *M. anisopliae* var *anisopliae* and of the desert locust, *Schistocerca gregaria* by *M. anisopliae* var *acridum* are consistent with this. In *Manduca* the appearance of acid phosphatase coincides with elevation of phosphate concentration up to the optimum for fungal growth. The reduction and finally the cessation of feeding in mycosed insects may reduce the availability of inorganic phosphate and further necessitate the use of organic phosphates. The hydrolysis of organic phosphates is of double benefit for the fungus in providing both carbohydrate and phosphate and the secretion of acid phosphatase later in mycosis is occurring when the trehalose (the other major carbon source) concentration is in decline (Phalaraksh et al., 2000).

The variety of organic phosphates in haemolymph may necessitate a corresponding suite of fungal phosphatases or a limited number of enzymes with broad substrate specificities.

In *M. sexta* infection the fungal acid phosphatase(s) secreted by *M. anisopliae* have broad substrate specificity and can break down several sugar phosphates.

Metarhizium anisopliae* utilises trehalose in the haemolymph of *Manduca

Insect haemolymph usually contains a high concentration of trehalose ranging from 4-20 mg/ml (Wyatt, 1967), and can be at very high levels, often exceeding 100mM in *M. sexta* (Thompson et al., 1990). Some other disaccharides eg. maltose, and sucrose have also been found in haemolymph of some species (Wyatt, 1967). In insects, as in other animals, glucose has a central place in carbohydrate metabolism. However, in the majority of species, the amount of free glucose is quite small, generally 10-fold lower than trehalose (Wyatt, 1967), because conversion of glucose to trehalose *in vivo* is rapid (Treherne, 1958, Clegg and Evans, 1961, Wyatt, 1967). The conversion of glucose to trehalose mainly occurs in the fat body and trehalose is released rapidly into the haemolymph with minimum storage; glycogen is the principal reserve carbohydrate.

Fungi can potentially utilise the trehalose in the haemolymph of insects by one of two strategies. The molecule may be transported intact across the plasma membrane or it may first be hydrolysed externally and the products (glucose) then transported inside (Jennings, 1995). The former strategy necessitates intracellular trehalase, and the latter one requires acid trehalase(s) and/or α -glucosidase(s). Disaccharides are impermeable substrates for some fungi eg. *Mucor* (Reyes and Ruiz-Herrera, 1972; Sorrentino et al., 1977), and the corresponding glucosidases must be external to the cell membrane (Sorrentino et al., 1977). Relatively few species assimilate trehalose without delay (Jenning, 1995),

suggesting trehalose is hydrolysed outside the cell membrane or that the disaccharide permease is induced rather than constitutive.

One of the extracellular α -glucosidases of *M. anisopliae* produced *in vivo* and *in vitro*, α -glucosidase1, is specific for trehalose and maltose and has a pH optimum similar to that of the haemolymph of *M. sexta*. So extracellular fungal α -glucosidase(s) may be one of the main hydrolases that hydrolyse trehalose in the haemolymph of the host insect.

Acid trehalase and neutral trehalase, may be also involved in the utilisation of trehalose in the haemolymph. Filamentous fungal acid trehalases are secretory proteins that have been localised to the cell wall (Hecher and Sussman, 1973; d'Enfert and Fontaine, 1977). Studies of knockout mutants of the acid trehalase genes in both *S. cerevisiae* and *Aspergillus nidulans*, and chemical mutants in *Neurospora crassa*, have revealed that these enzymes are involved in the assimilation of extracellular trehalose (Sussman *et al.*, 1971; Nwaka *et al.*, 1995; d'Enfert and Fontaine, 1997). In the present work, apart from α -glucosidase-1, two other trehalose hydrolysing enzymes were found in the haemolymph of infected *M. sexta* by IEF, which may well be acid trehalases. An intracellular neutral trehalase gene (*ntl1*) of *M. anisopliae* was expressed in liquid culture of cell-free haemolymph of *M. sexta* larvae and in the early stage of infection of *M. sexta* larvae. Thus both external hydrolysis of trehalose and direct uptake followed by internal hydrolysis may be operative for *Metarhizium* during growth in insect haemolymph.

There is a significant decline in haemolymph trehalose during mycosis (Phalaraksh *et*

2000) and this is matched by a large conspicuous increase in the concentration of glucose. Hydrolysis of sugar phosphates by the acid phosphatase must make a significant contribution to the elevation of glucose but the extracellular glucosidase and acid trehalases will also play a part. The increase in glucose concentration is probably very important for the development of fungus in haemolymph, because invariably glucose is one of the sugars most readily utilised by nearly all fungi (Jenning, 1995). If the fungal trehalose-hydrolysing enzymes are primarily cell wall bound enzymes, they would generate a high glucose concentration around the fungus, which could favour the fungus in the competition for glucose between pathogen and host.

***Metarhizium* hydrolases may be detrimental to the host**

The maintenance of a constant internal environment is very important for insects; this is made more difficult because of their small volumes, offering little storage and buffering capacity with which to smooth out almost constant fluctuations in supply and demand (Nijhout, 1994). However, fungal infection causes a complex pattern of physiological changes in host larvae including an elevation in glucose concentration and a lowering of trehalose. Variation in blood sugar in insects is important in the regulation of feeding behaviour (Simpson and Raubenheimer, 1993) and in the selection of food (Friedman et al., 1991). Indeed, feeding behaviour can be altered by artificially manipulating the blood sugar level (Friedman et al., 1991; Chapman and de Boer, 1995). Furthermore the concentration of trehalose plays a critical role in dietary selection and feeding behaviour, and is therefore important in regulating insect growth and development (Jones et al., 1981; Waldbauer and Friedman, 1991; Thompson, 1999). The average inter meal period of fifth

instar *M. sexta* larvae is 48 min (Timmins and Reynolds, 1992). Within 45 minutes of the end of a meal the haemolymph glucose levels decline rapidly (Timmins, 1988). The steepest concentration decline coincides with 48 minutes inter meal period. This suggests that glucose concentration may function in a feedback mechanism controlling the initiation of feeding. Therefore the large increase in haemolymph glucose may at least in part be responsible for the reduced feeding observed in *Manduca* larvae and indeed many other insects mycosed with *M. anisopliae* (Seyoum et al., 1994; Arthurs and Thomas, 2000).

A further consequence of the large increase in glucose concentration in the haemolymph of infected insects could be an increase in osmotic pressure. Changes in haemolymph osmotic pressure do contribute to the regulation of feeding in *Locusta migratoria*. However, osmotic pressure remained stable during mycosis (Xia *et al.*, unpubl obs.). The ability of the diseased insect to osmoregulate seems remarkable despite its poor condition.

The acid phosphatase(s) of *M. anisopliae* have a broad specificity against sugar phosphates. Thus, they may be able also to dephosphorylate phosphoproteins in haemolymph. This activity could cause host metabolic disorder and /or suppress the host defence response, since a number of proteins involved with both activities are activated by phosphorylation (Bettencourt et al. 1997; Kubota and Gay, 1995).

The study of acid phosphatases in locust haemolymph during mycosis revealed a complex picture. There was clear evidence for both host derived, probable defensive acid phosphatases, and fungal enzymes. It is interesting to note that chemical stimulation of the

immune system caused increased host enzyme but mycosis had a suppressive effect. This is a further example of *Metarhizium*'s ability to circumvent the host immune response. Host acid phosphatases were not evident in the study of *Manduca*. The reason is that the insect's enzyme has a different substrate specificity and was not demonstrated in the assays used for the *Metarhizium* enzyme.

The importance of fungal hydrolases in host mortality could be demonstrated unequivocally by transformation-mediated gene disruption or over-expression. At present, our knowledge of nutrient competition between insect and its fungal pathogens is poor and many questions remain unanswered; for example very little information exists on the roles of fungal extracellular hydrolases in utilisation of the nutrients in insects. This is surprising in view of the obvious importance of nutrient utilisation in infected insects. However, it is difficult to separate secondary effects of mycosis on host physiology from the direct effects of the fungus. Nevertheless, more detailed information is required on nutrient fluxes between host and pathogen.

Further experiments will be necessary to discover whether the impact of extracellular fungal hydrolases on haemolymph glucose is responsible for the decline in feeding in mycosed insects. However, if true this opens up a possible strategy for rational improvement of fungal pathogens for pest control. Up-regulation of fungal α -glucosidase could accelerate the decline in feeding and thus reduce crop damage. A precedent for this approach is provided by studies on baculoviruses. This is an important group of insect pathogenic viruses, in which deletion of the *egt* gene has significantly reduced feeding and

time to death of the host (O'Reilly and Miller, 1991).

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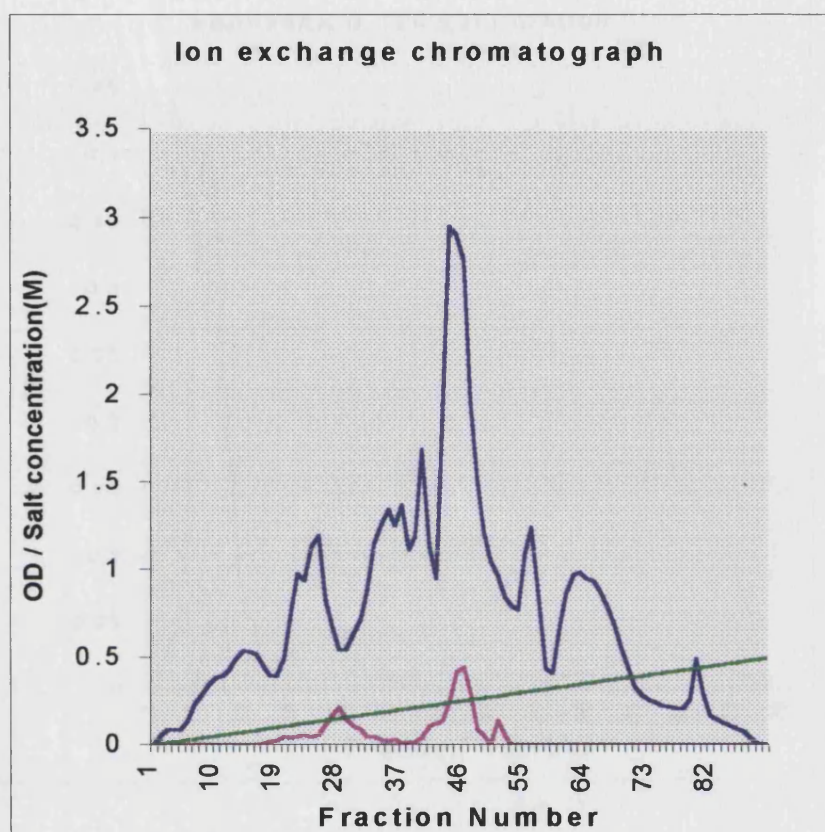
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APPENDIX

Appendix 1 Ion Exchange Chromatograph of *M. anisopliae* α -glucosidase on Q-sepharose



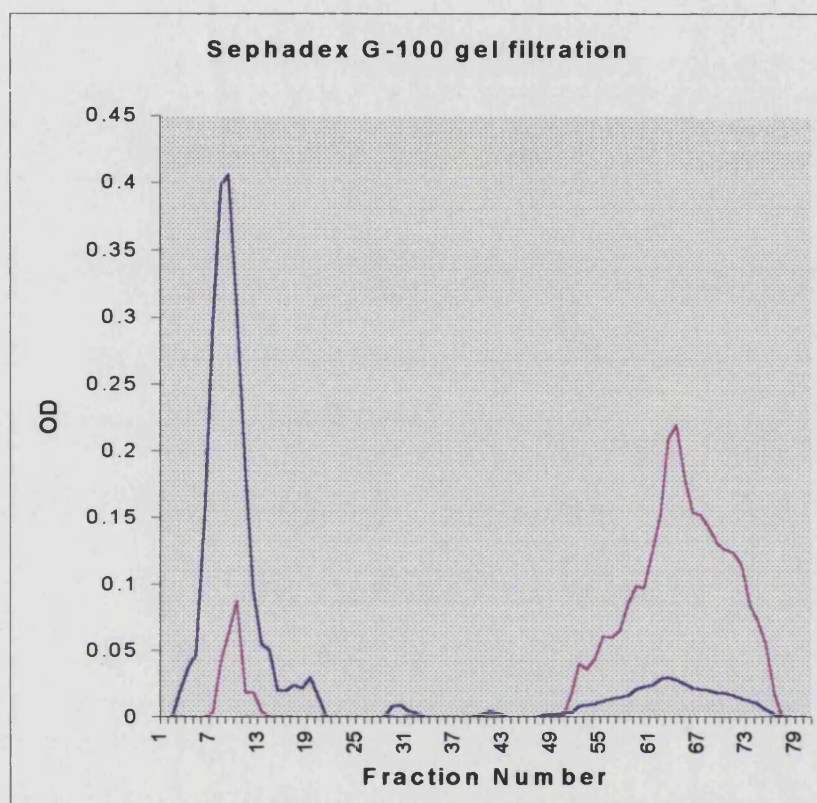
— OD280 : protein — OD520 : α -glucosidase activity

— Salt concentration (M)

The column (1x15cm) was equilibrated with 20mM acetate buffer pH 5.5. The column was washed with the same buffer and eluted with 0.0-0.5 M NaCl in buffer

(Flow rate: 1.5 ml/min, 4 ml/fraction).

Appendix 2 Sephadex G – 100 Gel Filtration of *M. anisopliae* α -glucosidase from Ion-exchange fractions(40-50)



— OD280 : protein

— OD520 : α -glucosidase activity

The column (2.4x90cm) was equilibrated with 20mM acetate buffer pH 5.5 (+0.1M NaCl). Proteins were eluted with same buffer at flow rate 1.5 ml/min, fractions were collected in 2.0 ml/fraction.